

HORMONAL REGULATION OF *the*
pathway
GLYCEROLIPID SYNTHESIS IN
BROWN ADIPOSE TISSUE.

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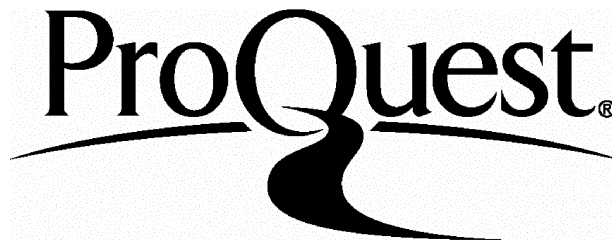
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ABSTRACT

Measurements were made of the activities of enzymes of glycerolipid synthesis in homogenates of interscapular brown adipose tissue. The enzymes studied were: mitochondrial and microsomal forms of glycerolphosphate acyltransferase (GPAT), Mg²⁺-dependant phosphatidate phosphohydrolase (PPH), fatty acyl-CoA synthetase (FAS) and monoacyl-glycerolphosphate acyltransferase (MGPAT). In normal animals cold-exposure (4°C) for 3-days increased all activities relative to tissue DNA. Mitochondrial GPAT showed a particularly marked increase (5-fold). Administration of the adipose tissue specific β_3 -adrenergic agonist (BRL-26830-A) resulted in a mimic of cold-induction in microsomal GPAT activity; no change was recorded for the other enzyme activities. Phenylephrine administration, either with BRL-26830-A or alone, resulted in reduction of activities of PPH, mitochondrial GPAT and MGPAT. Diabetes decreased mitochondrial GPAT activity but did not affect the increase in activity due to cold-exposure. Diabetes prevented the increase in activities of PPH and FAS due to cold-exposure. Hypothyroidism mimicked and enhanced the cold-induced increase in mitochondrial GPAT activity but did not affect the activity of PPH. Hyperthyroidism induced an approximate doubling of PPH activity but did not affect mitochondrial GPAT activity. Adrenalectomy resulted in an increase in mitochondrial GPAT activity which mimicked the effect of cold-exposure. PPH activity was increased by adrenalectomy and decreased by cold-exposure in the adrenalectomised animals. Cycloheximide administration prior to cold-exposure reduced the cold-induced increase in mitochondrial GPAT. These findings are relevant to signals that drive early events in mitochondriogenesis and cell proliferation in brown adipose tissue on exposure to cold.

Mitochondrial GPAT activity was observed in both outer and inner mitochondrial membranes from liver. Solubilisation of the mitochondrial GPAT activity resulted in an increase in K_m for glycerol-3-phosphate and sensitivity to N-ethylmaleimide. The solubilised activity was precipitated and stabilised in 12-15% polyethylene-glycol.

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Abbreviations.

ACTH	adrenocorticotrophic hormone
ADP	adenosine di-phosphate
ATP	adenosine tri-phosphate
BAT	brown adipose tissue
cAMP	cyclic adenosine mono-phosphate
cDNA	complementary deoxyribonucleic acid
CDP	choline di-phosphate
CoA	coenzyme A
CRF	corticotrophin releasing factor
CTP	choline tri-phosphate
DABA	3', 5-diaminobenzoic acid
DGAT	diacylglycerol acyltransferase
DHAP	dihydroxyacetone phosphate
DHAPAT	dihydroxyacetonephosphate acyltransferase
DIT	diet-induced thermogenesis
DNA	deoxy-ribonucleic acid
DTNB	5', 5-dithiobis-(2-nitro-benzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol bis-(β-aminoethyl ether) N, N, N', N'-tetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
FAS	long-chain fatty acyl-CoA synthase
FPLC	fast protein liquid chromatography
GDP	guanosine di-phosphate
GMP	guanosine mono-phosphate

GPAT	glycerolphosphate acyltransferase
GTP	guanosine tri-phosphate
HSL	hormone-sensitive lipase
LPL	lipoprotein lipase
MGPAT	monoacylglycerolphosphate acyltransferase
NA	noradrenaline
NAD	oxidised nicotinamide-adenine di-nucleotide
NEM	N-ethylmaleimide
NST	non-shivering thermogenesis
PDH	pyruvate dehydrogenase
PMSF	phenylmethane sulphonyl fluoride
PPH	phosphatidate phosphohydrolase
PTU	6-n-propyl-2-thiouracil
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
T₃	3', 5-triiodo-1-thyronine
T₄	thyroxine
TEF	thermic effect of food
TLC	thin-layer chromatography
VLDL	very-low-density lipoprotein
VMH	ventromedial hypothalamus
WAT	white adipose tissue

This Thesis is dedicated to the following people:-

My mother

My father

My step-mother

and All my family

Without whom the work presented below would never have appeared.

” There is a theory that states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.....

There is another theory which states this has already happened.”

**Douglas Adams (from the
Hitch-Hikers Guide to the
Universe Trilogy).**

I could not find a better description for what is generally found to be the case in scientific research. I hope the work presented in this thesis will move us a degree or two towards a better understanding of the role of brown adipose tissue in our present Universe.

J.R.D. Mitchell.

London, October 1992.

INTRODUCTION

CHAPTER ONE.

1.1. GLYCEROLIPID SYNTHESIS.

The glycerolipid synthesis pathway in mammals is vital in its provision of both triacylglycerols, for energy storage, and phospholipids essential for membrane production during cellular proliferation and mitochondriogenesis.

Regulation of the glycerolipid synthesis pathway (outlined in figure 1.1) has been extensively studied in both liver and white adipose tissue, but only a few studies from brown adipose tissue are known. Therefore, a concise resume of the known aspects of glycerolipid synthesis and its regulation in liver and white adipose tissue will be given prior to reviewing brown adipose tissue and glycerolipid synthesis in this tissue.

1.1A. Glycerolipid Synthesis in Liver and White Adipose Tissue.

The initial step in the utilisation of fatty acids in glycerolipid synthesis or β -oxidation is their conversion to the acyl-Coenzyme A (acyl-CoA) derivative. This conversion is catalysed by the enzyme long-chain fatty acyl-CoA synthetase (FAS). FAS is either ATP-dependant or GTP-dependant. However it is reported that in liver the ATP-dependant activity is 20-30 fold higher than that of the GTP-dependant activity (Van Tol, 1975) and the latter has no assigned physiological role to date. A number of different acyl-CoA synthetases, differentiated by their chain-length specificities, are present in the liver while white adipose tissue appears to contain only one enzyme with a broad chain-length specificity (Aas, 1971). ATP-dependant long-chain acyl-CoA synthetases are present in both microsomal and mitochondrial fractions, with the mitochondrial activity located in the outer-mitochondrial membrane (Aas, 1971; Nimmo, 1979). Both the mitochondrial and the microsomal enzymes are thought to have cytosolic active sites (Coleman and Bell, 1978; Hall and Saggerson, 1985; Heslar *et. al.*, 1990) and both utilise saturated and unsaturated fatty acids (Groot *et. al.*, 1976; Pande and Mead, 1968).

Long-chain acyl-CoA synthetase has been purified to homogeneity from rat liver microsomes and mitochondria, the purified enzyme having a subunit M_r of 76 kDa as determined by SDS-polyacrylamide gel-electrophoresis (Tanaka *et. al.*, 1979).

The first, or committed, step in the glycerolipid synthesis pathway is the formation

Figure 1.1 Outline of the Glycerolipid Synthesis Pathway.

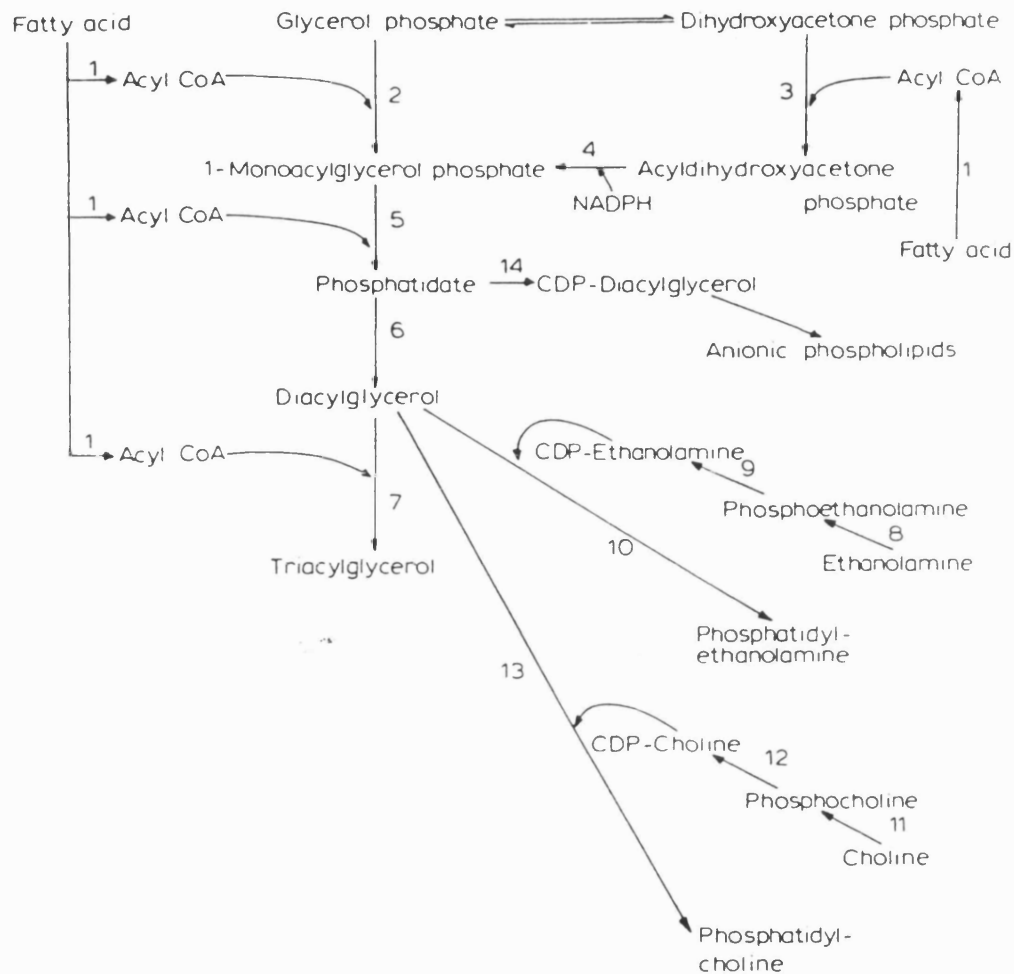


Figure 1.1 shows the general outline of the glycerolipid synthesis pathway. The main enzymatic steps and intermediates are shown. The enzymes are as follows; 1) fatty acyl-CoA (long-chain acyl-CoA) synthetase, 2) glycerolphosphate acyltransferase, 3) dihydroxyacetone phosphate acyltransferase, 4) acyldihydroxyacetone phosphate reductase, 5) mono-acylglycerolphosphate acyltransferase, 6) phosphatidate phosphohydrolase, 7) diacylglycerol acyltransferase, 8) ethanolamine kinase, 9) ethanolamine phosphate cytidyltransferase, 10) ethanolamine phosphotransferase, 11) choline kinase, 12) choline phosphate cytidyltransferase, 13) choline phosphotransferase, 14) CTP: phosphatidate cytidyltransferase.

of monoacyl glycerol-3-phosphate from acyl-CoA and either *sn*-glycerol-3-phosphate or dihydroxyacetone-phosphate. The formation using *sn*-glycerol-3-phosphate is catalysed by the enzyme glycerolphosphate acyltransferase (GPAT) which, is of great interest and importance since it is the committed enzyme for the pathway. In both liver and adipose tissue GPAT exists in two forms, one in the mitochondria and the other in the microsomal fraction. The distinction into different forms is based on differences in substrate specificity and inhibition by thiol-group reagents (Bates and Saggerson, 1979; Daae, 1973; Monroy *et. al.*, 1972). Mitochondrial GPAT will only use saturated acyl-CoAs as a substrate, whereas microsomal GPAT will utilise both saturated and unsaturated acyl-CoAs (Bremer *et. al.*, 1976; Daae, 1973; Haldar *et. al.*, 1979). In addition, microsomal GPAT shows less positional preference for placing of acyl-groups on the glycerol backbone (Haldar *et. al.*, 1979; Monroy *et. al.*, 1972). Mitochondrial GPAT also has a lower K_m for glycerol-3-phosphate and higher apparent affinity for palmitoyl-CoA than the microsomal form (Bates and Saggerson, 1979). The most distinctive difference between the two forms of GPAT is the effect of thiol-group reagents on the activity of the enzymes. Microsomal GPAT is completely inhibited by thiol-group reagents (such as N-ethylmaleimide) but the mitochondrial enzyme is unaffected (Bates and Saggerson, 1979; Lands and Hart, 1965; Monroy *et. al.*, 1972). In white adipose tissue the proportion of microsomal GPAT is greater than that in liver and its specific activity higher than that of the mitochondrial enzyme (Haldar *et. al.*, 1979; Saggerson *et. al.*, 1979; 1980; Schlossman and Bell, 1976).

Attempts to purify both mitochondrial and microsomal GPAT from rat liver have been made but only partial purification has been achieved (Monroy *et. al.*, 1973; Mok and McMurray, 1990). It has been proposed that the mitochondrial enzyme is located on the inner side of the outer-mitochondrial membrane (Heslar *et. al.*, 1985; Monroy *et. al.*, 1973; Nimmo, 1979). GPAT has been purified and characterised from *E. coli*. (Green *et. al.*, 1981; Scheidelar and Bell, 1986; 1989).

Monoacyl-glycerolphosphate can also be produced using dihydroxyacetone phosphate instead of glycerol-3-phosphate. This reaction is catalysed by

dihydroxyacetonephosphate acyltransferase (DHAPAT). DHAPAT is found in both liver and white adipose tissue, but shows different subcellular localisation in the two tissues. In liver DHAPAT is present mainly in peroxisomes but also occurs in microsomal and mitochondrial fractions, whereas in white adipose tissue DHAPAT is localised mainly in the microsomal fraction (Bates and Saggerson, 1979; Declerq *et. al.*, 1984; Hajra *et. al.*, 1979; Jones and Hajra, 1977; Saggerson *et. al.*, 1980; Schlossman and Bell, 1976). It is proposed that the microsomal GPAT and DHAPAT activities are present on one dual catalytic enzyme as both exhibit identical properties in terms of acyl-substrate, pH dependence, detergent inhibition and thiol-group reagent inhibition (Schlossman and Bell, 1976; 1977). The peroxisomal activity shows slight stimulation by thiol-group reagents and exhibits a partially latent activity in liver (Bates and Saggerson, 1979; Jones and Hajra, 1980).

The DHAPAT pathway contributes only 7% of total lipid synthesis in liver, the remaining 93% derives from the glycerol-3-phosphate pathway (Declerq *et. al.*, 1984). In white adipose tissue the glycerol-3-phosphate pathway exceeds that of dihydroxyacetone phosphate by at least 20-fold (Dodds *et. al.*, 1976). It appears that DHAPAT is essential for ether lipid synthesis (Declerq *et. al.*, 1984; Dodds *et. al.*, 1976). Although of interest, due to its low percentage contribution to overall glycerolipid synthesis, DHAPAT has not been studied in the work presented here.

Monoacylglycerol-3-phosphate is further acylated to produce phosphatidate, the reaction being catalysed by monoacylglycerolphosphate acyltransferase (MGPAT). This enzyme is predominantly microsomal in both liver and white adipose tissue (Okuyama *et. al.*, 1971; Saggerson *et. al.*, 1980; Yamashita *et. al.*, 1972). In liver it has been shown that MGPAT acylates with saturated acyl-CoA's preferentially at the 2-position of monoacylglycerol-3-phosphate (Okuyama *et. al.*, 1971). However once resolved away from microsomal GPAT, MGPAT will acylate 1-acylglycerol-3-phosphate with saturated and unsaturated acyl-CoA (Yamashita *et. al.*, 1972). Microsomal MGPAT activity in white adipose tissue is 7-fold higher than that of microsomal GPAT and 10-15 fold higher than

that of liver microsomal MGPAT (Saggerson *et. al.*, 1980).

Phosphatidate is dephosphorylated to produce diacylglycerol. This conversion is catalysed by the enzyme phosphatidate phosphohydrolase (PPH). The formation of diacylglycerol from phosphatidate directs glycerolipid synthesis into triacylglycerol, phosphatidylcholine and phosphatidylethanolamine production. Phosphatidate can also be converted to CDP-diacylglycerol and thence on to the anionic phospholipids. The anionic phospholipids include cardiolipin which is essential for inner mitochondrial membrane production.

Phosphatidate phosphohydrolase (PPH) is situated at an important branch point in the glycerolipid synthesis pathway and has been considered as a possible rate-limiting enzyme for the pathway as a whole. It has therefore been extensively studied in both liver (Bates and Saggerson, 1979; Brindley, 1988; Jamdar *et. al.*, 1984; 1991; Sturton and Brindley, 1980) and white adipose tissue (Saggerson, 1988; Saggerson *et. al.*, 1980; Taylor and Saggerson, 1986). PPH activity has been recorded in the mitochondrial, microsomal and cytosolic fractions of white adipose tissue (Jamdar and Fallon, 1973b; Saggerson *et. al.*, 1980; Taylor and Saggerson, 1986) and the enzyme is believed to be ubiquitous (Taylor and Saggerson, 1986). Two distinct enzyme activities have been shown in white adipose tissue, based on the dependance on Mg^{2+} for activity. Mg^{2+} -dependant activity has been shown to be both soluble and to be associated with the microsomal fraction of the cell, whereas the Mg^{2+} -independent activity seems to be associated only with the particulate fractions of the cell (Jamdar and Fallon, 1973b). The cytosolic PPH activity is almost exclusively Mg^{2+} -dependant and has been estimated to account for almost 70% of total Mg^{2+} -dependant activity (Jamdar and Fallon, 1973b; Saggerson *et. al.*, 1980). Mg^{2+} -independent activity has been estimated to account for only 20% of total PPH activity in the cell (Saggerson *et. al.*, 1980). Mg^{2+} -dependant PPH activity can be strongly inhibited by the thiol-group reagent N-ethylmaleimide (NEM), is heat labile and susceptible to attack by proteolytic enzymes (Jamdar *et. al.*, 1984). Mg^{2+} -

dependant PPH has a lower K_m for phosphatidate than the Mg^{2+} -independent activity (Jamdar and Fallon, 1973b; Jamdar *et. al.*, 1984). Ca^{2+} ions inhibit both PPH activities (Lawson *et. al.*, 1981).

The liver the two forms of PPH show identical properties to the white adipose tissue enzymes. Recent reports, suggest that the Mg^{2+} -independant PPH activity is present predominantly in plasma membranes. This activity is not inhibited by NEM, is stable in the presence of proteolytic enzymes and is heat stable (Jamal *et. al.*, 1991). It is proposed that this form of PPH is involved in signal transduction. Reports of PPH activity involved in signal transduction in other tissues support this additional role for PPH (Bishop *et. al.*, 1990; Rossi *et. al.*, 1990; Welsh *et. al.*, 1990).

The specific activities of the central enzymes involved in glycerolipid synthesis are 5-10 fold higher in white adipose tissue than those in liver, reflecting the high capacity for triacylglycerol synthesis in this adipose tissue (Bates and Saggerson, 1980; Billah *et. al.*, 1989; Yamashita *et. al.*, 1973; Young and Lynen, 1969).

1.1B. Short-Term Regulation in Liver and White Adipose Tissue.

A major component of glycerolipid synthesis (especially in white adipose tissue) is triacylglycerol production. The regulation of esterification has been studied in detail and appears to be under tight hormonal control. The control of esterification can be applied to all the aspects of the glycerolipid synthesis pathway if it is assumed that the regulatory enzyme for the pathway is one of the central enzymes mentioned below. Although no definite identification has been made this would seem likely.

In white adipose tissue, insulin and noradrenaline (norepinephrine, NA) have antagonistic effects on triacylglycerol synthesis (reviewed by Saggerson, 1985). It has yet to be elucidated whether the stimulatory actions of insulin are distal to the activation of glucose transport (Simpson and Cushman, 1986). It has been shown, by using [^{14}C]-fructose or [3H]- H_2O , that insulin stimulates production of glyceride glycerol from the above precursors (Sooranna and Saggerson, 1975). In general lipolytic hormones increase

the rate of esterification in adipose tissue by increasing the supply of fatty-acids (Grahn and Davies, 1980), while decreasing the capacity of the esterification pathway, thus preventing excess futile cycling (Saggerson, 1972, 1985). Insulin stimulates fatty acyl-CoA production and simultaneously increases esterification of fatty acyl-CoA's with glycerol-3-phosphate derived from increased glucose metabolism. Insulin also inhibits lipolysis, combining with the above to lead to reduced levels of fatty acyl-CoA in white adipose tissue. In contrast β -adrenergic agonists such as noradrenaline increase fatty acyl-CoA levels in adipocytes (Page-Penuelas *et. al.*, 1987).

The hormonal effects on the central glycerolipid synthesis enzymes are summarized below. The summary pays particular attention to regulation in white adipose tissue. Where appropriate the known regulation in liver (and other tissues) will be detailed.

FAS is inhibited by adrenaline, the inhibition being potentiated by glucose and antagonised by insulin and propranolol (Sooranna and Saggerson, 1978). Inhibition of FAS appears to be parallel with stimulation of lipolysis by noradrenaline and is reversed by insulin or propranolol (Hall and Saggerson, 1985).

The committed enzyme in the pathway, namely glycerolphosphate acyltransferase (GPAT), is an obvious choice for a possible regulatory enzyme and as such its regulation has been studied in detail in white adipose tissue. Adrenaline inhibits GPAT activity, the inhibition is potentiated by glucose and antagonised by propranolol (*c. f.* FAS) (Sooranna and Saggerson, 1976). As incubation with fatty acids had no significant effect on GPAT activity it was proposed that the adrenaline inhibition was a direct effect and not secondary to effects on lipolysis (Sooranna and Saggerson, 1976b). The inhibition by noradrenaline on both mitochondrial and microsomal forms of GPAT is by a lowering of the V_{max} of the enzyme (Rider and Saggerson, 1983). Initial studies did not show any direct effect on GPAT activity by insulin (Evans and Denton, 1977; Sooranna and Saggerson, 1976; 1978) but insulin was shown to block the noradrenaline-induced inhibition (Rider and Saggerson, 1983). Recently Vila and Farese (1991) showed insulin increased GPAT activity in white adipose tissue and postulated a phosphoinositol-linked phospholipase C mediation. Insulin

has also been shown to increase GPAT activity in myocytes (Vila *et. al.*, 1990).

DHAPAT activity appears to be regulated in a very similar manner to that of GPAT, supporting the hypothesis of a single enzyme containing dual-activity (Horie *et. al.*, 1990; Schlossman and Bell, 1976; Sooranna and Saggerson, 1979).

There appear to be no reports on the hormonal regulation of MGPAT in white adipose tissue or liver to date.

Mg²⁺-dependant PPH has also been put forward as a possible regulatory enzyme for glycerolipid synthesis. It catalyses the formation of diacylglycerol from phosphatidate which occurs at an important branch-point in the glycerolipid synthesis pathway. In liver changes in triacylglycerol synthesis during different physiological states are reflected in the translocation of, (between cytosol and particulate fractions) and activity of Mg²⁺-dependant PPH. This supports PPH's proposed role as the rate-limiting enzyme for the glycerolipid synthesis pathway in this tissue. Also in liver, triacylglycerol synthesis is linked to phosphatidylcholine production, as the export and transport of triacylglycerol from the liver is via very-low-density lipoproteins (VLDL). VLDL's consist of 20% phosphatidylcholine and 60% triacylglycerol, so co-ordinated production of both is required in liver. Phosphatidylcholine levels are regulated by CDP-choline levels, which are regulated by the enzyme CDP-phosphocholine cytidyltransferase (CDP:PCT). The regulation of CDP:PCT in liver is remarkably similar to that of PPH, with translocation from cytosol to microsomal fractions when activated, also being seen (Pelech and Vance, 1984; Vance and Pelech, 1984). This demonstrates a direct link between the regulation of triglyceride production and phospholipid production should the two be required together. PPH has also been shown to translocate to mitochondria, allowing triacylglycerol, phosphatidylcholine and phosphatidylethanolamine production from mitochondrially-synthesised phosphatidate (Freeman and Mangiapane, 1989).

In white adipose tissue the inhibition of PPH activity by adrenaline or noradrenaline was blocked by insulin or propranolol, although insulin alone has no effect on PPH activity

(Cheng and Saggerson, 1978a,b). Adrenaline decreases PPH activity by a cAMP-mediated response (Haghighi *et. al.*, 1990). It should be noted that only the Mg^{2+} -dependant PPH is hormonally regulated and is thought to be the only form important in the glycerolipid synthesis pathway (Jamdar and Fallon, 1973a,b). The physiological role of Mg^{2+} -independent PPH is yet to be elucidated in white adipose tissue, but may be involved in signal transduction in liver (Jamal *et. al.*, 1991).

Insulin is reported to have no effect on diacylglycerol acyltransferase (DGAT) activity but antagonises the inhibition induced by adrenaline (Sooranna and Saggerson, 1978).

The mechanisms underlying these short-term hormonally-induced changes have yet to be elucidated.

1.1C. Long-Term Regulation in Liver and White Adipose Tissue.

The long-term regulation of the glycerolipid synthesis pathway has been studied extensively in both liver and especially in white adipose tissue. Thus, in the following resume the known regulation in white adipose tissue will be emphasized, with that known in liver given where appropriate.

In white adipose tissue the capacity for glycerolipid synthesis is reduced during starvation (Angel and Roncari, 1967; Harper and Saggerson, 1976; Saggerson, 1972a), streptozotocin-induced diabetes (Saggerson and Carpenter, 1987) and during aging (Jamdar and Osbourne, 1982; Jamdar *et. al.*, 1986).

Decreased activities of FAS (Lawson *et. al.*, 1981), GPAT (Angel and Roncari, 1967; Jamdar and Osbourne, 1982; Sooranna and Saggerson, 1979; Taylor and Saggerson, 1986), DHAPAT (Sooranna and Saggerson, 1979) and Mg^{2+} -dependant PPH (Jamdar and Osbourne, 1982; Moller *et. al.*, 1977) have been recorded in white adipose tissue during starvation. Microsomal GPAT has not been shown to change during starvation (Lawson *et. al.*, 1981).

Refeeding of starved animals resulted in restoration of GPAT and PPH activity

levels (Jamdar and Osbourne, 1982).

Streptozotocin-induced diabetes results in decreased activities of FAS, MGPAT, Mg²⁺-dependant PPH (Saggerson and Carpenter, 1987) and GPAT (Saggerson and Carpenter, 1987; Taylor and Saggerson, 1986). All activities are restored by 2 day or 2 h. treatment with insulin (Saggerson and Carpenter, 1987; Taylor and Saggerson, 1986)

GPAT and PPH activities are also reduced in hypothyroid white adipose tissue, with restoration of control levels of activity after 3 days of thyroxine administration (Taylor and Saggerson, 1986). Recently, administration of tri-iodothyronine (T₃) to guinea pigs was shown to result in elevated lung GPAT activity, the elevation being dependant upon transcription and translation (Mukherjee *et. al.*, 1990).

Increasing age leads to decreased activities of GPAT, DGAT and PPH (Jamdar *et. al.*, 1984, 1986). The activity of MGPAT does not change with respect to aging and is unaffected by cell size (Jamdar *et. al.*, 1986). GPAT, DGAT and PPH all have greater activities in larger adipocytes, which parallels the increased triacylglycerol synthesis in these cells over the smaller adipocytes (Jamdar *et. al.*, 1981; 1986).

In liver physiological stress leads to an increased supply of fatty acids. This is accompanied by increased β -oxidation due to a lowering of malonyl-CoA levels which relieves the inhibition of, and thus increases the activity of, carnitine palmitoyltransferase (McGarry and Foster, 1980). However the capacity for β -oxidation in the liver can, during conditions such as diabetes, be exceeded leading to increased hepatic glycerolipid synthesis (Murthy and Schipp, 1979; Woods *et. al.*, 1981). PPH activity levels are elevated in diabetes (Murthy and Schipp, 1979; Woods *et. al.*, 1981) and starvation (Kinnula *et. al.*, 1978; Mangiapane *et. al.*, 1973; Vavrecka *et. al.*, 1969). The increased activity of PPH in diabetes is reversed by treatment with insulin (Murthy and Schipp, 1979; Woods *et. al.*, 1981). PPH has also been shown to increase in diabetic heart where glucagon causes translocation of PPH to the particulate fractions (Schoonderwoerd *et. al.*, 1990). PPH is also elevated during regeneration of liver after partial hepatectomy, indicating the

importance of the glycerolipid synthesis pathway during cell proliferation and tissue growth (Tijburg *et. al.*, 1991).

The activities of the other enzymes central to the glycerolipid synthesis pathway appear to be either unchanged or decreased during long-term stress in liver. Hepatic GPAT is decreased during starvation (Aas and Daae, 1971; Bates and Saggerson, 1979; Vavrecka *et. al.*, 1969), diabetes (Bates and Saggerson, 1979) and lipid peroxidation (Thomas and Poznansky, 1990). FAS and DHAPAT also decrease during starvation (Bates and Saggerson, 1979; Mangiapane *et. al.*, 1971).

Overall, in white adipose tissue elevated levels of catecholamines relative to insulin decrease the capacity of the glycerolipid synthesis pathway by decreasing the activities of FAS, GPAT, DHAPAT and PPH.

In liver similar conditions result in increased glycerolipid synthesis (especially triacylglycerol) associated with elevated PPH activity but unchanged or decreased GPAT activity. It would appear, therefore, that in liver the most likely candidate for the regulatory enzyme for glycerolipid synthesis is PPH and not the first committed enzyme GPAT.

Only a small amount of work has been carried out on this pathway in brown adipose tissue. Prior to reviewing that work a brief introduction to brown adipose tissue, its functions and regulation will be given below.

1.2. BROWN ADIPOSE TISSUE.

Thermogenic processes can be classified under two major headings:-

Obligatory thermogenesis; which is the heat associated with metabolic processes such as ingestion, digestion, absorption and storage of food (known as the thermic effect of food or TEF). Also included in this category is the energy expended during growth, lactation and tumour growth. Changes are small and generally appear to be regulated by thyroid hormones (Himms-Hagen, 1989).

Facultative thermogenesis accompanies processes which are switched on and off relatively quickly by the nervous system. Exercise and cold-induced shivering are examples

of this category. Non-shivering thermogenesis, produced by activation of brown adipose tissue, is also a facultative thermogenic response.

The primary function of brown adipose tissue (BAT) is to produce metabolic heat without measurable muscle contraction (*i.e.* without shivering), either via cold-induced non-shivering thermogenesis (NST), or diet-induced thermogenesis (DIT) (Reviews by Trayhurn and Nicholls, 1986 and Himms-Hagen, 1989; 1990).

BAT is thought to be exclusive to mammals as, since its first description (Gessner, 1551), it has only been found in mammals. It is possible to speculate that the appearance of BAT may have coincided with the development of the homeothermes (organisms which can maintain their core temperature within a few degrees centigrade despite large environmental temperature fluctuations). The homeothermes include the mammals. Poikilothermies, in contrast to homeothermes, are the organisms which are unable to maintain a constant core temperature (*e.g.* reptiles) and do not possess BAT.

Cold-induced NST in BAT is induced by a number of conditions:-

1) Cold exposure; animals (such as rats, mice or hamsters) are placed at a temperature sufficiently below their thermoneutral temperature (the temperature at which no thermoregulation is apparent) for a short period of time (*e.g.* rats placed at 4°C for 4h.).

2) Cold acclimation (sometimes referred to as adaptation); animals are placed in a cold environment (see above) for a long period of time. This treatment is likely to be more physiological as, in the wild, animals would be exposed to decreasing temperatures which then remain low for an extended period *e.g.* winter. The laboratory version of long-term cold exposure is to subject the animal to 4°C for a period of not less than 3 days and up to 3 months. During acclimation to cold BAT undergoes recruitment, where tissue DNA and protein increases and total BAT mass is elevated (this is not usually seen in cold exposure).

3) Arousal from hibernation; Initial raising of the core temperature from the low level during hibernation is thought to be achieved by NST in BAT. Once shivering is possible this form of heat production becomes the major one (Nedergaard and Cannon, 1984 for review).

4) In newborn mammals (reviewed Nedergaard and Cannon, 1992); There are a number of different times at which BAT is developed and/or activated in newborns. NST in BAT buffers the cold shock experienced at birth and when venturing into the environment for the first time. Altricial (nest-dependant) newborns are born relatively under-developed. They are blind, helpless, without fur or other insulation and are without functional BAT, which develops just prior to the newborn exiting the nest, where it encounters its first cold shock. It is however, possible to recruit BAT from the moment of birth . Altricial newborns includes the rat and mouse. Precocial newborns, including the guinea-pig and lamb, are very well developed at birth (they are usually fully independently mobile within a few minutes of birth) and have fully functional, often activated, BAT at birth. The development and activation of BAT *in utero* is the most interesting aspect of these newborns. Immature newborns are those which are born in an extremely under-developed state where recruitment of BAT (or any independent thermoregulation) is not possible for at least several days post-partum. This group is small but includes the marsupials, hamster and possibly the ground squirrel (Nedergaard *et. al.*, 1986; Nedergaard and Cannon, 1992).

What is clear is that until the capacity for other thermoregulatory processes (*e.g.* fur and/or shivering) is developed, the major thermoregulatory site *in vivo* in newborns is BAT. Most larger mammals (*e.g.* humans) lose the majority of their functional BAT soon after maturation of all thermoregulatory processes. However both the hibernators and the small mammals, such as rats and mice, retain most of their functional BAT, making them ideal models for studying BAT structure, function and metabolism.

The remaining form of thermogenesis (DIT) is, as its name suggests, induced by overeating and is used to maintain a lean body weight (reviewed by Rothwell and Stock, 1986; Stock, 1989).

Both NST and DIT are switched on and off in response to changes in demand for heat or fuel combustion. The cellular mechanisms involved in NST and DIT are thought to be the same, although the central control may be via different areas of the brain (Rothwell,

1989).

Thus, in contrast to white adipose tissue (WAT), BAT utilises excess dietary, or stored energy to produce heat. The heat produced needs to be transported around the entire body to have an overall thermic effect. This is achieved by transport of heat by circulating blood, which passes through the various BAT depots. BAT depots are primarily situated near the major blood vessels (*e.g.* around the aorta, above the heart and in the kidney area) and other depots (such as interscapular and cervical) are extremely well supplied with blood, the flow of which has been shown to increase dramatically during NST (Kuroshima *et. al.*, 1967). Figure 1.2 shows the distribution and amount of BAT in the rat.

BAT contains, in addition to mature brown adipocytes, interstitial cells and preadipocytes (capable of differentiating into mature brown adipocytes during cold acclimation) and endothelial cells (also recruited during acclimation to cold) (Bukowiecki *et.al.*, 1982; Bukowiecki, 1986; Geloan *et. al.*, 1988).

Brown adipose tissue differs white adipose tissue, in a number of key ways:-

1) White adipocytes contain more than twice the amount of lipid (80-90%) than brown adipocytes.

2) Mature adipocytes generally have a unilocular triglyceride droplet in white adipose tissue which is larger than the multilocular triglyceride droplets in active brown adipocytes. When BAT is quiescent brown adipocytes tend to take on the appearance of white adipocytes in terms of their triglyceride droplet(s).

3) The noradrenaline (NA) content of white adipose tissue is lower than that of BAT but innervation appears to be distributed in a similar manner in both tissues (Stock and Westermann, 1963; Rosell and Belfrage, 1979).

4) Maximal blood flow through white adipose tissue is lower (0.25 ml/min/g tissue) than that through BAT (7-20 ml/min/g tissue). The variation seen in maximal blood flow through BAT is apparently due to a dependance upon which BAT depot is studied (Foster and Frydman, 1978).

5) The mitochondria in BAT are larger, more numerous and have a larger cristae

Figure 1.2. Distrubution of Brown Adipose Tissue in the Rat.

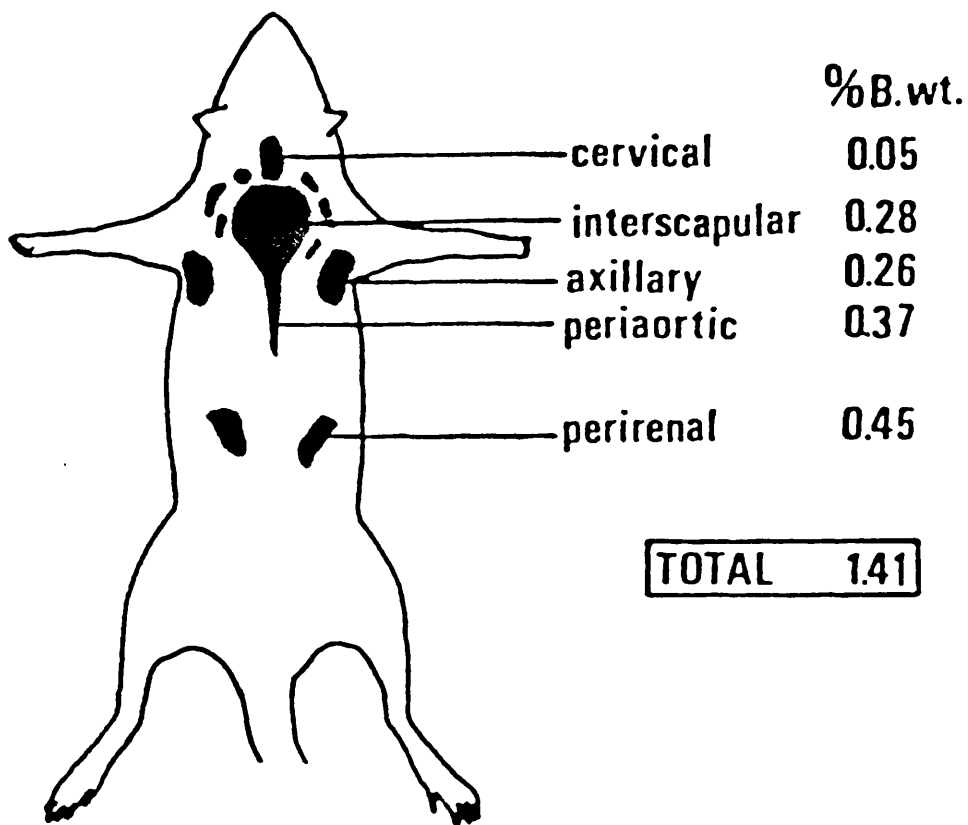


Figure 2 shows the distrubution and % of total body weight of brown adipose tissue depots in the rat. The depot of brown adipose tissue which is the most extensively studied is the interscapular depot. Figure 2 is adapted from Rothwell and Stock (1986), with permission.

surface area than that found in white adipose tissue. More importantly, and most specifically, is the exclusive occurrence of the nucleotide-binding protein, thermogenin (known also as uncoupling protein, GDP-binding protein or 32kDa protein), in BAT mitochondria. It is the regulated uncoupling property of this protein (uncoupling respiration from ATP production in the mitochondria by allowing the dissipation of the proton gradient across the inner mitochondrial membrane) which allows the combustion of substrate yielding heat, estimated to be as much as 300 watts/kg tissue (Nedergaard and Lindberg, 1982). The specificity of the occurrence of thermogenin in BAT provides an accurate criterion by which the tissue can be identified *i.e.* the occurrence of, or ability to produce thermogenin identifies the tissue as BAT (see section 1.2A. for more details).

Brown adipocytes have the capacity to communicate directly with adjacent cells via gap junctions (bridge-like contacts between two continuous plasma membranes). The area of gap junctions increases in parallel with increasing thermogenic activity, suggesting an important role for intercellular signaling in BAT during thermogenesis (Schneider-Picard *et. al.*, 1980; 1984). Mitochondrial membranes and plasma membranes are often seen close to one another, with small vesicles below invaginations in the plasma membrane. It is suggested that these structures could be involved in fuel supply from extra-cellular sources (Greco-Perotto *et. al.*, 1987a, b).

1.2A. Thermogenin and the Mechanism of Thermogenesis:

In mitochondria β -oxidation of fatty acids is coupled, via respiration, to production of ATP. The production of ATP is dependant upon the presence of a proton gradient across the inner mitochondrial membrane which drives the synthesis of ATP in a process known as oxidative phosphorylation.

When BAT is stimulated, the proton gradient across the inner mitochondrial membrane is dissipated and the mitochondria are uncoupled (*i.e.* oxidation occurs without appropriate ATP production). Thus heat is produced instead of ATP (*i.e.* thermogenesis occurs). The BAT specific protein thermogenin is responsible for the dissipation of the proton gradient (for reviews on thermogenin see Trayhurn and Nicholls, 1986;

Klingenburg, 1990). Thermogenin, as some of its alternative names suggest (see section 2), is a 32kDa protein, which binds nucleotides (such as GDP or ADP) and is a proton translocator (Nicholls *et. al.*, 1986). Thermogenin also binds fatty acids and acyl-CoA esters, with acyl-CoA esters thought to bind at the same site as nucleotides (Katiyar and Shargo, 1991). The protein has been shown to span the inner membrane of BAT mitochondria several times, with the C-terminal end projecting from the outer surface (cytosolic facing surface) of the membrane (Aquila *et. al.*, 1985; Eckerskorn and Klingenburg, 1987). Recently Winkler and Klingenburg (1992) have shown the adenosine binding site on thermogenin to be localised at a hydrophilic channel in the tripartite structural domain spanning the membrane and the site can be accessed from both the cytosolic and matrix surfaces of the membrane. Binding of nucleotide to the binding site confers total inhibition of proton translocation on thermogenin (Nicholls, 1976a,b,c). The binding of the nucleotide (GDP experimentally and probably ADP or ATP physiologically) appears to be highly pH dependant (Klingenberg, 1984; 1988). The small rise in intracellular pH, seen when BAT is stimulated (Giovannini *et. al.*, 1988), may be sufficient to displace any bound nucleotide and thus activate thermogenin and thermogenesis. Thermogenin also appears to translocate halide ions in a nucleotide regulated manner (Jezek *et. al.*, 1990). Although generally expected as being exclusive to BAT, thermogenin messenger RNA (mRNA) has been reported in cold-acclimated (4°C for 4wks) and newborn, rat liver (Shinohara *et. al.*, 1991), but the levels shown were very much lower than those seen in BAT. This report is unique and therefore lacks confirmation.

Various methods of acute regulation of thermogenin have been forwarded, all of which involve the removal of nucleotide bound to thermogenin when BAT is in the quiescent state. Change in pH (see above), competitive binding and displacement of nucleotide by acyl-CoA esters and decreases in physiological levels of nucleotides (ATP and/or ADP) via a putative ATPase, have all been suggested. However all the above lack any substantial corroboration. The most probable acute regulation of thermogenin is by binding of fatty acids to the protein, thereby increasing proton conductance resulting in

reduction in membrane potential (Nicholls *et al.*, 1986; Rial *et al.*, 1983). Fatty acids are thought to be the major fuel for thermogenesis and have been demonstrated to be increased intracellularly during stimulation of thermogenesis (see sections 1.2C. and 1.2G) thus fatty acids would act as both substrate and signal for increased thermogenesis. Fatty acids have been shown to directly interact with and regulate thermogenin in brown adipocytes (Bukoweicki *et al.*, 1981; 1986; Cunningham *et al.*, 1986) and isolated mitochondria (Locke *et al.*, 1982a,b; Rial *et al.*, 1983), but not in reconstituted phospholipid vesicles (Klingenburg and Winkler, 1985; 1986; Strieleman *et al.*, 1985a,b; Strieleman and Shargo, 1985).

Chronic regulation of thermogenin involves changes in both protein and mRNA levels (Nedergaard and Cannon, 1987; Jacobsson *et al.*, 1986; 1987; Peachey *et al.*, 1988). Cold-acclimation results in increased levels of mRNA for thermogenin (but with decreased stability of the mRNA) and increased thermogenin protein levels, which is in excess of the increases in mitochondrial and tissue protein also seen (Ashwell *et al.*, 1983; Desautels *et al.*, 1978; Jacobsson *et al.*, 1986; 1987; Sundin *et al.*, 1987). Recently an enzyme-linked immuno-sorbant assay (ELISA) for thermogenin has been developed and experiments using this technique also show increased thermogenin levels in cold acclimation (Chan and Swamainathan, 1991). Increased synthesis of thermogenin mRNA is a very rapid response (apparent at 15 mins in the nucleus) and is maximal only 12-24h. after the start of cold stress (Jacobsson *et al.*, 1986; 1987; Ricquier *et al.*, 1986). Increased levels of thermogenin mRNA have also been observed in BAT of "cafeteria-diet" fed animals (*i.e.* DIT stimulated) (Falcou *et al.*, 1985) with similar increases being inducible by infusion of noradrenaline (NA) and by chronic treatment with β_3 -adrenergic specific agonists, such as BRL 26830A (see section 1.2C.) (Cunningham and Nicholls, 1987; Ricquier *et al.*, 1984; 1986). Although generally thought to be essentially a β -adrenergic mediated response, there is growing evidence that both α - and β -adrenergic receptors are involved in the regulation of thermogenin gene expression (Jacobsson *et al.*, 1986; Rehnmark *et al.*, 1990). There appears to be an absolute requirement for tri-

iodothyronine (T_3) for thermogenin mRNA production and translation (Giralt *et. al.*, 1990; Houstek *et. al.*, 1990; Reiter *et. al.*, 1990). T_3 has no effect on thermogenin in the absence of sympathetic stimulation suggesting a permissive role only for T_3 (Silva, 1988). Lactation causes a specific loss of thermogenin protein which is reversed on weaning (Trayhurn and Jennings, 1987), also diabetes results in a loss of thermogenin (Jamal and Saggerson, 1988a; Seydoux *et. al.*, 1984). A rapid reversal of cold-induced changes in thermogenin is seen on the return to a warm environment, which is associated with reduction (and possibly cessation) of sympathetic stimulation of BAT (Himms-Hagan, 1989).

Changes in capacity for thermogenesis in BAT is usually assessed by measuring the extent of GDP-binding to thermogenin in isolated mitochondria (GDP is used as it is not translocated into the mitochondria as is ADP). However GDP-binding cannot be used as an accurate measurement of levels of thermogenin, due to the phenomenon of acute "unmasking" of binding sites observed by numerous researchers (see Himms-Hagan, 1989 for references). Thus the extent of GDP-binding gives an estimation of thermogenic capacity of BAT, but not an accurate estimation of thermogenin content in the tissue (with the possible exception of long-term stimulation) (Himms-Hagan, 1989).

1.2B. Central Regulation of Thermogenesis in Brown Adipose Tissue.

The central control (*i.e.* control originating from, and within, the brain) of thermogenesis has been studied since the first reports that destruction of the ventromedial hypothalamus (VMH) resulted in animal obesity (Han *et. al.*, 1965; Stellar, 1954; central control reviewed by Rothwell, 1989). The hypothalamus as a whole has been considered to be the brain region controlling autonomic functions, such as feeding behaviour and thermoregulation, for some considerable time. Various effects of stimulation of the VMH on BAT have been reported, indicating a close association between the two. Shimazu and Tckahashi (1980) showed increased lipogenesis in BAT due to electrical stimulation of the VMH but not other brain regions. Perkins *et. al.* (1981) indicated increased thermogenesis

due to stimulation of the VMH (by measurement of temperature change in the tissue region only), and this has been confirmed by demonstration of :-

- 1) Increased blood flow to BAT (Iwai *et. al.*, 1987).
- 2) Increased noradrenaline turnover in BAT (Saito *et. al.*, 1987).
- 3) Increase in overall resting O₂ consumption in BAT (Atrens *et. al.*, 1985).

Reciprocally, inhibition of thermogenesis in BAT has been demonstrated following VMH destruction or inhibition (Carlisle *et. al.*, 1988; Han, 1968; Hogan *et. al.*, 1982; Nijima *et. al.*, 1984; Seydoux *et. al.*, 1981). The involvement of the ventromedial hypothalamic nuclei and paraventricular nuclei in the regulation (usually positive) of BAT has been reported (Amir, 1990a, b; Freeman and Wellman, 1987). Stimulation of BAT thermogenesis by stimulation of the posterior hypothalamus has been reported (Amir, 1990c). Other brain regions postulated to be involved in BAT regulation include; the supraoptic nucleus (known to be involved in arousal from hibernation) (Rothwell 1989), the preoptic area (Holt *et. al.*, 1987; Freeman and Wellman, 1987) and the preoptine region (Rothwell *et. al.*, 1983; Shibata *et. al.*, 1987).

Intrahypothalamic injection of glucose increases BAT activity (Sakaguchi and Bray, 1987; 1988). Injection of the inhibitory analogue of glucose (2-deoxyglucose) into the same region results in inhibition of BAT activity (Arase *et. al.*, 1987). Uptake of radiolabeled 2-deoxyglucose into various areas of the brain after preoptic or hypothalamic cooling has also been demonstrated (Marimoto and Murakami, 1985). Activation of the VMH results in increased glucose uptake into BAT (Sudo *et. al.*, 1991) which, with the above evidence, suggests that glucose could act centrally to direct its own distribution and metabolism. Insulin is also thought to have an unclarified central effect on BAT regulation (Rothwell and Stock, 1988; Sakaguchi and Bray, 1987). The central noradrenergic pathways are also implicated in BAT regulation (Liebowitz, 1978; Sahakian *et. al.*, 1983), with changes in noradrenaline concentration, turnover and ratio of noradrenergic: serotonergic pathways considered to be of primary importance in body temperature regulation (Myers, 1972). This is perhaps not surprising as the major physiological effector

for BAT thermogenesis is noradrenaline released from the local sympathetic nerves. The involvement of the serotonergic pathway in BAT regulation is supported by reports from Rothwell and Stock (1987); Rothwell (1989) and by the demonstration that 5-hydroxytryptamine (5-HT) levels are elevated in BAT during cold exposure and during the initial few days after birth (Bertin *et. al.*, 1991).

Corticotrophin-releasing factor (CRF) has been reported to be a potent activator of BAT metabolism and thermogenesis (LeFruvre *et. al.*, 1987), and is thought to be responsible for increased thermogenesis and BAT activity after adrenalectomy (Busbridge *et. al.*, 1990; Holt *et. al.*, 1988; Kim and Romsos, 1990). CRF acts on the medial preoptic area and results in increased sympathetic nervous activity in interscapular BAT (Ewawa *et. al.*, 1990). The effect of adrenalectomy may be confined to obese animals (Walker and Ramsos, 1992).

Thyrotropin-releasing hormone (TRH) has been shown to cause arousal from hibernation (Stanton *et. al.*, 1980) and to have a potent effect on BAT thermogenesis (Griffiths *et.al.*, 1988).

Other brain peptides which have been reported to increase thermogenic activity in BAT include; interleukin 1 β and 1 α (Dascombe *et. al.*, 1987) and cholecystinin quatrapeptide (CC4) (Rothwell 1989).

1.2C. Local Control of Brown Adipose Tissue and Thermogenesis.

The metabolic activity of BAT is under both hormonal and sympathetic-mediated adrenergic regulation, both acutely (*e.g.* cold exposure) and chronically (*e.g.* cold-acclimation).

1.2C.i. Acute Regulation.

BAT plasma membranes contain both α - and β -adrenergic receptors and receptors for insulin, glucagon, adenosine, adrenocorticotrophic hormone (ACTH), thyrotrophin, growth hormone and nicotinic acid. Associated with acute (and chronic) changes in thermogenesis in BAT are changes in blood flow to the tissue, essential for substrate

supply to the tissue and heat distribution from the tissue.

The most important acute physiological regulation of BAT thermogenesis is by catecholamines released from sympathetic nerve endings (Nicholls and Locke, 1984; Bukowiecki, 1984). The central controls described in section 1.2B have been shown to affect the firing rate of the sympathetic nerves which supply BAT (Himms-Hagan, 1989). Noradrenaline released from these nerves binds to both α - and β -adrenoreceptor types resulting in increases in respiration and elevated heat production. It has been reported that α -adrenoreceptor stimulation accounts for approximately 20% of the maximal noradrenaline-stimulated thermogenesis, with β -adrenergic stimulation accounting for the remaining 80% of the maximal response (Mohell *et al.*, 1981; 1983; Schimmel *et al.*, 1983). The binding of noradrenaline results in a biphasic membrane depolarisation. The initial rapid phase is α -induced and precedes the stimulation of metabolism. The later phase is β -induced and occurs after metabolic indices have reached their maximum (reviewed by Girardier and Seydoux, 1986).

Both α_1 and α_2 -adrenoreceptors have been identified in BAT. The nature of the β -adrenoreceptor in BAT has recently been proposed to be a mixture of novel β_3 -adrenoreceptors (Arch, 1989) but this classification remains contentious. The presence of an atypical β -adrenergic receptor in BAT has been suspected since the development of novel β -agonists, such as BRL-26830A, which are thought to be BAT specific, do not conform to being β_1 or β_2 -receptor agonists (Arch *et al.*, 1984; Holloway *et al.*, 1991; Cawthorne *et al.*, 1992) and are now thought to be β_3 -receptor specific. A cDNA for the β_3 -receptor has recently been cloned and characterised from rat BAT (Muzzin *et al.*, 1991), and mRNA levels for the receptor have been shown to be regulated by noradrenaline and β_3 -specific agonists such as BRL-26830A. β_3 -adrenoreceptor mRNA has been reported in white adipose tissue as well as BAT (Granneman and Lahnens, 1992).

The second messengers involved in the adrenergic mediated changes in BAT metabolism are thought to be; cyclic adenosine-monophosphate (cAMP) and Ca^{2+} . cAMP

levels rise due to increased adenylate cyclase activity, (β -adrenergic mediated response) and elevation of cytosolic Ca^{2+} is via the phosphoinositol second messenger system (α_1 -adrenergic mediated response). Also there is likely to be some inhibition of the adenylate cyclase system via the activation of the α_2 -adrenergic receptors (see section 1.2C.ii.) and possibly by adenosine.

1.2C.ii. α -Adrenergic Regulation.

Stimulation of the α_1 -adrenergic receptors results in increased production of two second messengers; diacylglycerol and inositol triphosphate (IP_3), via an increased phosphatidylinositol biphosphate cycle (Mohell *et. al.*, 1984; Nanberg and Putney, 1986; Schimmel *et. al.*, 1986; 1987).

The largest, and most immediate, response to α_1 -stimulation is a rise in Ca^{2+} release from intracellular stores (Connolly *et. al.*, 1984; Nedergaard *et. al.*, 1986) and translocation of protein kinase C to the plasma membrane (Barge *et. al.*, 1988). The observed increase in K^+ efflux is probably a response to the increase in Ca^{2+} (Connolly *et. al.*, 1984; Nanberg *et. al.*, 1984). The activation of a diacylglycerol-dependant protein kinase C results in the activation of Na^+/H^+ exchange and the induced Na^+ entry leads to further Ca^{2+} release (Connolly *et. al.*, 1984; Nanberg *et. al.*, 1985; Nedergaard *et. al.*, 1986). The altered levels of K^+ and Na^+ activate the Na^+ , K^+ -ATPase, resulting in an increase in ATP hydrolysis. The α_1 -response is ATP dependant, as the altered ion gradients need energy to be restored (Mohell *et. al.*, 1987). Arachidonic acid has also been reported to be released due to stimulation of the α_1 -receptor (Schimmel, 1988).

The raised Ca^{2+} level has been reported to affect the activities of pyruvate dehydrogenase phosphate phosphatase, NAD^+ -isocitrate dehydrogenase and oxoglutarate dehydrogenase, in the mitochondria (McCormack and Denton, 1977; McCormack, 1985a, b).

Stimulation of the α_2 -adrenoreceptors may cause partial inhibition of adenylate

cyclase and lipolysis (Dominquez *et. al.*, 1986; Skala and Shaikh, 1988; Skala *et. al.*, 1988).

α_2 -mediated increases in cyclic GMP (cGMP) have been reported in foetal rat BAT, and the known role of cGMP in the development process indicates a possible stimulatory role for α_2 -receptor-mediated response in the trophic response, seen in BAT, during chronic stimulation (Dominquez *et. al.*, 1986; Sundin and Fain, 1983) (see section 1.2D.).

1.2C.iii. β -Adrenergic Regulation.

BAT contains β_3 -adrenergic receptors (see 1.2C.) coupled to an adenylate cyclase system in the plasma membrane. Noradrenaline interacts with these receptors resulting in increased cAMP levels via activation of the adenylate cyclase. G_s alpha has been shown to be associated with the adenylate cyclase (Granneman and McKenzie, 1988). The β -stimulated processes accounts for 80% of noradrenaline-induced maximally stimulated mitochondrial respiration and therefore 80% of thermogenesis. Uncouplers of mitochondrial respiration do not increase maximal respiration rate, however sub-maximal stimulated respiration may not be totally uncoupled from ATP synthesis (Mohell *et. al.*, 1987).

The major consequences of β -adrenoreceptor stimulation, via cAMP-dependant protein kinase, are:-

1) the activation of hormone-sensitive lipase (HSL) (see section 1.2G.i.), leading to increased lipolysis, resulting in increased fatty acid levels (Bukowiecki, 1984; Nicholls and Locke, 1984)

2) Activation of lipoprotein lipase (LPL) (see section 1.2G.ii.), which also leads to increased intracellular levels of fatty acids (Cameheim *et. al.*, 1984).

Other consequences of β -adrenergic stimulation are; inactivation of acetyl-CoA carboxylase (Gibbins *et. al.*, 1985), activation of pyruvate dehydrogenase (Gibbins *et. al.*, 1985) and activation of phosphofructokinase-1 (Sale and Denton, 1985).

β -adrenergic stimulation of isolated cells has been demonstrated to lead to increased

Na⁺ influx (Connolly *et. al.*, 1986) and increased Ca²⁺ influx (Connolly and Nedergaard, 1988). The later is a consequence of fatty acid-induced uncoupling of mitochondria, the former a more direct cAMP-mediated effect (Connolly *et. al.*, 1986; Connolly and Nedergaard, 1988).

1.2C.iv. Acute Affects of Thyroid Hormones on Brown Adipose Tissue.

BAT contains thyroxine 5'-deiodinase, the enzyme which converts thyroxine (T₄), secreted from the thyroid gland, to 3,5,3'-tri-iodothyronine (T₃), which is the thermogenically active hormone (Leonard *et. al.*, 1983; Silva and Larsen, 1983). Acute exposure to cold can lead to an extremely large (up to 100-fold) increase in enzyme concentration, which is regulated by noradrenaline (Jones *et. al.*, 1986; Kopecky *et. al.*, 1987; Leonard *et. al.*, 1983; Silva and Larsen, 1983). BAT has nuclear receptors for T₃ and these receptors are reported to be saturated within 4h. of cold exposure, during which time intracellular levels of T₃ are elevated (Bianco and Silva, 1987; 1988; Burgi and Burgi-Saville, 1986; Gaikwad *et. al.*, 1990). BAT can be affected by circulating or intracellularly generated T₃ (Silva and Larsen, 1985). The increased T₃ levels observed in BAT after noradrenaline stimulation of the tissue are involved in tissue hypertrophy (see section 1.2D.i.), and have a permissive role in the acute thermogenic phase (Seydoux *et. al.*, 1986; Sundin *et. al.*, 1984). The increase in 5'-deiodinase in response to cold requires both α - and β -adrenergic stimulation, acting in a synergistic fashion (Raasmaja, 1990). T₃ does not appear to be thermogenically active by itself, requiring simultaneous sympathetic stimulation of BAT. This has proved difficult to assess properly, as hypothyroidism is associated with suppression of the sympathetic nervous system in BAT (Knehans and Romsos, 1984; Rothwell and Stock, 1984; Sundin, 1981). The stimulation of lipogenesis by T₃, seen in liver and white adipose tissue, is not observed in BAT (Sugden *et. al.*, 1983). Hypothyroidism results in increased lipogenesis in BAT (Baht and Saggerson, 1988a, b; Freake and Oppenhiemer, 1987).

1.2C.v. Control of Blood Flow Through Brown adipose Tissue.

Blood flow through BAT is essential for provision of fatty acids (obtained via the action of lipoprotein lipase on circulating chylomicrons and very-low-density lipoproteins) required for thermogenesis once intracellular stores are exhausted and for distributing the heat produced in the tissue around the body. Also transport of any exported products, in addition to heat, would be via circulating blood.

Blood flow through BAT is increased enormously during stimulation of thermogenesis and is directly related to the thermogenic state of the tissue, although the direct effect of noradrenaline on BAT blood vessels appears to be vasoconstrictive (Foster, 1984; 1986). There is a very low flow rate through BAT at thermoneutrality, which is increased at "room" temperature (usually approximately 21°C) and is maximal at 4°C (Foster and Frydman, 1978a, b; 1979; Wickler *et al.*, 1984; 1986). The direct release of noradrenaline from sympathetic nerve endings would appear to be the route of control of blood flow through BAT (and almost all the effects of noradrenaline on BAT), as circulating concentrations of noradrenaline are insufficient (Depocas *et al.*, 1978; Foster *et al.*, 1982). Several candidates for a putative vasodilator have been forwarded, with adenosine as the most likely candidate (Schimmel and McCarthy, 1984; 1986; Szillat and Bukowiecki, 1983). As yet no direct evidence for adenosine as the vasodilator exists (Ma and Foster, 1984), but the extreme sensitivity of adrenergic responses to adenosine suggests only a very small amount of total adenine nucleotide would be required for blood flow regulation (Schimmel *et al.*, 1987). There is an α_1 -receptor stimulation-mediated potentiation of noradrenaline action on β -adrenergic receptors which increases blood flow (Ma and Foster, 1984) and evidence suggests that α_1 -receptor stimulation can override the adenosine inhibition of β -receptor mediated lipolysis and thermogenesis (Schimmel and McCarthy, 1984; 1986). This effect is probably due to an α_1 -receptor mediated potentiation of cAMP release (Ma and Foster, 1984). It has been suggested that blood vessels and brown adipocytes are innervated by two different subpopulations of autonomic neurones (Cannon *et al.*, 1986) and that a dual-control process of BAT thermogenesis and

vasodilation exists (Benzi *et. al.*, 1988).

1.2D. Chronic Regulation of Brown Adipose Tissue.

Chronic stimulation of BAT (*e.g.* cold stress for any period exceeding approximately 3-7 days) results in tissue hypertrophy. This tissue hypertrophy includes hyperplasia, increased mitochondrial mass and a parallel increase in thermogenic capacity of the tissue (increase in thermogenin concentration per mitochondria and per cell). The increase in mitochondrial mass may precede the increase in total tissue and exceeds tissue growth, leading to increased mitochondrial concentration in the tissue. Increases in the key components of the thermogenic response and tissue growth, for example; thermogenin (see section 1.2A.), lipoprotein lipase (LPL), thyroxine 5'-deiodinase (see section 1.2C.iv. and below), glucose transporters and gap junctions, are seen. Changes in BAT during hypertrophy are generally greater with long-term cold stress than with long-term over-eating (cafeteria diet for long periods). Hypertrophy is primarily mediated by the sympathetic nervous system, but hormonal regulation has been demonstrated in conjunction with the sympathetic control.

Chronic lack of stimulation of BAT can lead to atrophy of the tissue. This atrophy can be induced by denervation but not by sympathetic nervous system suppression, indicating that other factors are additionally involved in the regulation of tissue atrophy. As atrophy progresses mitochondrial mass and thermogenin levels decrease. Many obese animal models exhibit non-functional, atrophied BAT, a condition which has been suggested as a primary reason for the development of the obese state (reviewed by Himms-Hagan, 1989). The development of BAT specific drugs, which result in re-activation of BAT from the atrophied state, may be of immense therapeutic value in the treatment of obesity in humans. With this goal in mind understanding the mechanisms and early events in BAT hypertrophy and the signals involved in atrophy are of great interest and significance.

An important point to note is that species variation of BAT hypertrophy is evident, for example the Syrian hamster (a hibernator) shows a distinct photosensitivity in BAT

growth which is not seen in the rat or mouse (Himms-Hagan, 1989). For the purposes of this thesis the following resume of BAT hypertrophy and atrophy will be confined (in the majority) to reported observations from work with rats and mice.

1.2D.i. Hypertrophy and Hyperplasia of Brown Adipose Tissue.

Hyperplasia is induced by chronic cold stress (Bukowiecki *et al.*, 1982; 1984; Geleon *et al.*, 1988), cafeteria diet (Rothwell and Stock, 1982a) and sucrose feeding (Bukowiecki *et al.*, 1983). The hyperplasia induced by the above can be mimicked by chronic noradrenaline or isoproterenol (β -adrenergic specific agonist) administration but not by chronic administration of phenylephrine (α -adrenergic specific agonist) (Geleon *et al.*, 1988), and can be blocked by simultaneous chronic administration of propranolol (β -adrenergic antagonist) (Rothwell and Stock, 1982b). Increased mitosis in endothelial cells has been noted as a prelude to the increase in the capillary bed which accompanies hypertrophy of BAT (Bukowiecki *et al.*, 1982; 1984; Geleon *et al.*, 1988).

Changes in gene expression and protein levels of thermogenin (Jacobsson *et al.*, 1986; 1987; Ricquier *et al.*, 1986) and lipoprotein lipase (LPL) (Carneheim *et al.*, 1984; 1988; Mitchell *et al.*, 1989) have been shown to accompany tissue growth. LPL is the enzyme which provides the essential fatty acid supply from the circulating chylomicrons and VLDLs' enabling increased thermogenesis and phospholipid production essential for membrane production. The changes in activity and mRNA levels of LPL are mediated by β -adrenergic pathways (Carneheim *et al.*, 1984; 1988). Dietary effects on LPL are unclear but a possible role for insulin in regulation of changes in LPL activity is suggested (Bertin *et al.*, 1985; Carneheim and Alexson, 1989; Granneman and Wade, 1983; Smolin *et al.*, 1986). Increases in thermogenin mRNA and protein levels are thought to require both α - and β -adrenergic stimulation (Jacobsson *et al.*, 1986; 1987; Rehnmark *et al.*, 1990), the presence of insulin (Geleon and Trayhurn, 1990a, b; Yoshioka *et al.*, 1989) and the presence of T_3 (see sections 1.2A. and 1.2C.iv.). Thyroxine 5'-deiodinase protein and activity increases in parallel to tissue growth and this response is abolished by denervation. However thyroxine 5'-deiodinase activity returns to its previous levels once

tissue growth is complete whereas thermogenin activity remains high (Eley and Himms-Hagan, 1989), supporting the suggestion that this enzyme and its product (T_3) play a permissive role only in BAT hypertrophy.

Changes in the plasma membrane of hypertrophied BAT include; increased translocation of glucose transporters to the cell surface and increased numbers of these transporters with associated changes in their properties (Greco-Perotto *et al.*, 1987a, b). Also seen is an increased density of gap junctions and α_1 -adrenergic receptors, paralleling the recruitment level of the tissue (Raasmaja *et al.*, 1984; Raasmaja and York, 1988; Raasmaja, 1990). It is also proposed that the responsiveness of adenylate cyclase to noradrenaline is enhanced in cold-acclimated BAT (Granneman *et al.*, 1985; Granneman and McKenzie, 1988).

What is abundantly clear is that during long-term stimulation of BAT large changes in membrane (plasma and mitochondrial in particular) levels and composition occur. The increased membrane production would require increased phospholipid production (amongst other things) which implies increased flux through the glycerolipid synthesis pathways, giving an additional role (other than just replenishment of utilised triglyceride) to this aspect of BAT metabolism.

1.2D.ii. Atrophy of Brown Adipose Tissue.

Atrophy of BAT occurs when stimulation of the tissue is removed (surgical denervation) or chronically suppressed (lactation and/or exercise-training).

As it has been established that sympathetic nervous stimulation of the tissue is the most important aspect of induction of BAT hypertrophy, and thermogenesis, it is not surprising that denervation of the tissue results in rapid atrophy. The atrophy induced in this manner is manifested as decreased total tissue protein and total mitochondrial mass. After a lag period of approximately 1 day, thermogenin is selectively reduced (Desautels *et al.*, 1986; Park and Himms-Hagan, 1988). A rapid decline in thyroxine 5'-deiodinase is seen (Park and Himms-Hagan, 1988) with a decline in LPL also apparent (Billington *et*

al., 1987). The reductions seen are larger and more rapid when the tissue has been cold stressed prior to denervation (Himms-Hagan, 1989).

Physiological conditions resulting in BAT atrophy, leading to similar changes to those seen in denervated tissue, are; starvation, lactation, diabetes and exercise-training.

Fasting (starvation) results in loss of total and mitochondrial protein (Trayhurn and Jennings, 1986; 1988), with conflicting observations on whether thermogenin is (Trayhurn and Jennings, 1986; 1988) or is not (Desautel and Dulos, 1988) selectively lost.

BAT remains relatively unchanged during pregnancy until just prior to parturition (Champigny and Hitier, 1987; Villarroya *et al.*, 1986). Lactation induces an atrophic response associated with a loss of total and mitochondrial protein and selective reduction in thermogenin levels (Andrews *et al.*, 1986; Ashwell *et al.*, 1983). LPL and thyroxine 5'-deiodinase activities are also reduced (Champigny and Hitier, 1987; Giralt *et al.*, 1986). These changes are thought to be associated with a reduction in sympathetic nervous activity in BAT (Trayhurn and Wusteman, 1987; Villarroya *et al.*, 1987).

Long-term diabetes induces loss of total protein, mitochondrial protein and a specific reduction in thermogenin protein, with associated reduction in GDP-binding but no reduction in the cell numbers in BAT (Bartness *et al.*, 1986a, b; Jamal and Saggerson, 1988; Seydoux *et al.*, 1983). Reduced activities of LPL and thyroxine 5'-deiodinase are also observed (Seydoux *et al.*, 1984; Shibata *et al.*, 1987; Silva and Larsen, 1986).

Exercise-training may or may not affect BAT in warm-adapted animals (Himms-Hagan, 1989) but exercise-training does prevent cold-induced changes in BAT (Arnold *et al.*, 1986; Arnold and Richard, 1987).

Atrophy of BAT induced by a return to thermoneutrality after cold-acclimation, displays one important difference to the atrophy described above:- namely a reduction in total DNA level. Also reductions in protein and mRNA levels are more rapid (Desautels *et al.*, 1986; Leblanc and Diamond, 1988; Peachey *et al.*, 1988; Reichling *et al.*, 1987).

1.2E. Endocrine Influences.

1.2E.i. Insulin.

Insulin has many, varied, effects on BAT metabolism, either directly or centrally. It is generally thought that most insulin effects are coupled to changes in sympathetic nervous system activity in BAT.

Central administration of insulin is reported to have opposing effects on BAT depending upon where the site of injection is in the brain and the time of day that the hormone was administered (Rothwell, 1989; Sakaguchi *et. al.*, 1988; Sakaguchi and Bray, 1988; Young, 1988).

Insulin receptors have been identified in BAT plasma membranes (Knott *et. al.*, 1990) and insulin's direct effects on BAT are thought to include regulation of glucose transport and metabolism (Rothwell and Stock, 1987; Himms-Hagan, 1989), and a permissive role in cold-induction of thermogenesis and increased thermogenin levels (Geleon and Trayhurn, 1990a, b; Yoshioka *et. al.*, 1989). Insulin may also play a part in differentiation of precursor cells into mature brown adipocytes (Klaus *et. al.*, 1991).

Diabetic-induced atrophy (see section 2.Dii) can be partially reversed by chronic β -adrenergic agonist treatment (Arch *et. al.*, 1986) and some aspects reversed by insulin treatment (Gualberto and Saggerson, 1989) although this recovery could be the result of insulin stimulating the sympathetic nervous system in BAT (Jamal and Saggerson, 1988).

Increased sensitivity of BAT to insulin is seen on exposure and acclimation to cold, which is associated with the tissue hypertrophy seen during long-term cold stress (see section 1.2D.i.) (Vallerand *et. al.*, 1987). Obesity has been suggested to be linked to insulin resistance in BAT. However, there is also an associated reduction in the sympathetic nervous system in BAT during obesity. This makes it difficult to clarify whether insulin resistance is just an aspect of the reduced sympathetic drive, or a separate part of the signals involved in the reduction of thermogenesis and atrophy of BAT, associated with obesity (Himms-Hagan, 1989).

1.2E.ii. Thyroid Hormone.

The role of T_3 in the regulation of acute thermogenesis and BAT hypertrophy have been described in sections 1.2C.iv. and 1.2D.i. It is worth repeating that T_3 effects require

sympathetic stimulation of the tissue and the effect of T_3 is thought to be a permissive one (Silva, 1988). Changes in thyroxine 5'-deiodinase lag behind those seen for thermogenin mRNA and are thought to be involved in permissive regulation of gene transcription and protein synthesis (Kaplon and Young, 1987; Jones *et. al.*, 1986). Changes in BAT seen in hypothyroidism are similar to those seen in diabetes in that oxygen utilisation and GDP-binding are reduced, indicating decreased thermogenic capacity (Jamal and Saggerson, 1988a, b; Woodward and Saggerson, 1989). This could be an indication that insulin is involved in regulation of thyroid hormone levels in BAT, via control of thyroxine 5'-deiodinase activity which is seen to be very low and unresponsive to cold stress in the diabetic state (Silva and Larsen, 1986). Recently it has been reported that decreased thyroxine 5'-deiodinase activity affects thermogenin but not LPL mRNA levels (Rieter *et. al.*, 1990), which suggests that substrate supply and oxidation may become dissociated during hypothyroidism and diabetes. This idea is supported by the observation that β -oxidation of fatty acids from lipolysis in BAT is reduced in both diabetes (Jamal and Saggerson, 1988) and hypothyroidism (Woodward and Saggerson, 1989), whereas the esterification potential of brown adipocytes doubles during hypothyroidism (Baht and Saggerson, 1988; Blenneman *et. al.*, 1992). Hypothyroidism has also been shown to result in a loss of β -adrenergic receptor numbers and reduction in the receptor mRNA levels (Revelli *et. al.*, 1991).

Hyperthyroidism results in increased GDP-binding and increased O_2 -consumption which may be a reflection of increased thermogenin levels or increased "unmasking" of nucleotide binding sites (Woodward and Saggerson, 1989).

From the above it can be seen that the control of thermogenesis and hypertrophy could be a combined, highly sensitive, regulation involving adrenergic, thyroid and insulin mediation of BAT.

1.2E.iii. Glucocorticoids.

The effect of glucocorticoids on BAT has been studied mainly in obese animal

models, since it was demonstrated that adrenalectomy reversed the impairment of BAT thermogenesis in these animals. (York, 1989; Himms-Hagan, 1989, for obesity reviews). Since then adrenalectomy has been demonstrated to slightly increase the thermogenic capacity in warm-adapted lean animals (Busbridge *et. al.*, 1990). Diet affects the degree of control of BAT due to adrenal status (Rothwell *et. al.*, 1984; Smith and Ramsos, 1985), with high carbohydrate diets leading to the most pronounced effects.

The central effects of adrenalectomy or adrenal suppression have been described previously (see section 1.2B.).

Direct effects of glucocorticoids or adrenalectomy are varied and involve suppression or reactivation of BAT respectively. The effects are manifested in changes in protein, mitochondrial and GDP-binding characteristics (Holt *et. al.*, 1983; Freedman *et. al.*, 1986).

Restoration of thermogenic capacity in *fa/fa* rats and *ob/ob* mice has been shown to be associated with increased sympathetic drive to BAT (Van de Tuig *et. al.*, 1984; York *et. al.*, 1985) adding evidence to the hypothesis of a complex, integrated control of BAT centering around the sympathetic nervous system.

1.2E.iv. Glucagon.

Chronic administration of glucagon induces a cold-induction like response in BAT (Billington *et. al.*, 1987). Glucagon levels are markedly increased during cold exposure and cold acclimation, and an important, though undefined, role for glucagon in BAT regulation has been suggested. However the levels of glucagon required to induce changes in BAT are not seen physiologically, therefore the physiological role of glucagon in BAT remains unclear (Himms-Hagan, 1989).

1.2E.v. Pituitary Hormones.

The effects of the pituitary hormones, if any, on BAT appear to be inhibitory as hypophysectomized rats, with replacement T₄ and corticosterone, exhibit increased BAT responsiveness to cold stress, which is dependant upon innervation (Himms-Hagan, 1989; Holt *et. al.*, 1988; Rothwell and Stock, 1985). It has been suggested that the effect stated

above is probably due to CRF acting centrally (see section 1.2B.) to increase BAT sympathetic nervous system activity (Himms-Hagan, 1989).

1.2F. Carbohydrate Metabolism in Brown Adipose Tissue.

There is now general agreement that glucose is not a major energy source for stimulated thermogenesis in BAT (Isler *et al.*, 1987; Lopez-Soriano and Alemany, 1986; Saggerson *et al.*, 1988; Smith *et al.*, 1986; Wilson *et al.*, 1987). Also it has been demonstrated that glucose is not the best substrate for lipogenesis in BAT (Saggerson *et al.*, 1988). BAT does utilise glucose and, under some stimulated circumstances, BAT can be a major site of glucose disposal (Cooney *et al.*, 1987; Cooney and Newsholme, 1984; Vallerand *et al.*, 1989; Young *et al.*, 1985). The stimulated utilisation of glucose in BAT is under both hormonal (insulin) and adrenergic (noradrenaline) control.

Insulin increases BAT glucose utilisation both *in vivo* (Cooney *et al.*, 1987; Ferre *et al.*, 1986; James *et al.*, 1986; Penicaud *et al.*, 1987) and *in vitro* (Ebner *et al.*, 1987; Saggerson *et al.*, 1988). The glucose response shows increased sensitivity in the cold acclimated state (probably due to increased insulin sensitivity) (Smith *et al.*, 1986; Vallarand *et al.*, 1987; 1989) and there is a selective increase in numbers of, and translocation of, glucose transporters in the plasma membrane (Greco-Perotto *et al.*, 1987a, b).

Noradrenaline and other β -specific adrenergic agonists increase glucose utilisation and transport (Cooney *et al.*, 1985; Ma and Foster, 1986; Marette and Bukowiecki, 1990; Wilson *et al.*, 1987). Noradrenaline activates key enzymes involved in glucose utilisation in BAT, such as phosphofructokinase (Sale and Denton, 1985) and pyruvate dehydrogenase (PDH) (Gibbins *et al.*, 1985). PDH is extremely sensitive to ATP and will only be active when ATP levels are low (Denton *et al.*, 1984). Insulin also activates PDH in BAT (Denton *et al.*, 1984; McCormack and Denton, 1977). It is possible that the noradrenaline-mediated activations may be secondary to the fatty acid uncoupling of the mitochondria (Himms-Hagan, 1989; Marette and Bukowiecki, 1990).

The role of glucose metabolism in BAT is not clear. The major product of glucose metabolism in BAT is lactate (Denton *et al.*, 1984; Isler *et al.*, 1987; Ma and Foster, 1986). During maximal stimulation of thermogenesis 88% of glucose uptake is re-exported as lactate or pyruvate, with 33% at submaximal thermogenesis (Ma and Foster, 1986). This accelerated glycolysis could serve as a means of providing ATP, via substrate level phosphorylation, to compensate for the lack of mitochondrial oxidative phosphorylation during the uncoupled state (Himms-Hagan, 1989). Glycolysis can also provide acetyl-CoA, glycerol-3-phosphate and NADPH, via the pentose-phosphate pathway (glucose-6-phosphate dehydrogenase is present at high activity levels in BAT (Cooney and Newsholme, 1986)). The production of these substrates for lipogenesis and esterification of fatty acids could be a role for glucose utilisation. However, as the major product of glucose utilisation is lactate, the provision of the above intermediates is likely to be of less importance than the provision of ATP (Ma and Foster, 1986). It has also been suggested that the pyruvate produced from glucose could be used to provide an anaplerotic replenishment of tri-carboxylic acid cycle (TCA) intermediates (Cannon and Nedergaard, 1979). The reduced NAD produced during glycolysis could be used in the production of glycerol 3-phosphate, via dihydroxyacetone phosphate, which can be used in the esterification of fatty acids to produce triacylglycerol and phospholipids.

It has been hypothesised that BAT could be involved in a modified "Cori cycle" (similar to that seen between anaerobic muscle and liver) with lactate exported from BAT, transported to the liver, converted back to glucose and returned to BAT for glycolytic provision of ATP. The ATP produced in this manner would provide a means of overcoming the loss of ATP production from the uncoupled mitochondria during thermogenesis. Thus the liver would play an important supportive role in maintaining BAT metabolism and thermogenesis (Himms-Hagan, 1989).

1.2G. Lipid Metabolism in Brown Adipose Tissue.

The immediate and major fuel for thermogenesis in BAT is fatty acids, as glucose has been confirmed to be only a minor energy source (in terms of thermogenesis) in this

tissue (see section 1.2F.).

The sources of fatty acid required for enhanced, uncoupled β -oxidation in BAT mitochondria, are; intracellular lipolysis of triacylglycerol stores (via the action of hormone-sensitive lipase) in the acute phase, extracellular circulating triglycerides from chylomicrons and very-low-density lipoproteins (VLDLs) (via the action of lipoprotein lipase) during chronic stimulation of thermogenesis, as well as *de novo* synthesis.

1.2G.i. Hormone-Sensitive Lipase.

Holm *et. al.*, (1988) reported a protein in BAT which was immunologically similar to hormone-sensitive lipase (HSL) from white adipose tissue. It is assumed that the regulatory mechanism controlling HSL in BAT is the same as that seen in white adipose tissue (Bukowiecki, 1986), involving phosphorylation/ dephosphorylation of the enzyme. The total amount, in terms of protein, of HSL is increased in cold-stimulated BAT, but at a level parallel to that of total tissue protein, indicating the increase in HSL protein is not specific (unlike the changes seen with thermogenin and lipoprotein lipase). In BAT a direct relationship between lipolysis and noradrenaline concentration exists only at low levels of noradrenaline. The level of noradrenaline required to half-maximally stimulate thermogenesis *in vitro* is 10-fold lower than that required to half-maximally stimulate lipolysis (Jamal and Saggerson, 1988a)

The capacity for lipolysis in BAT appears, therefore, to greatly exceed that of thermogenesis. When BAT is stimulated by pharmacologically high levels of noradrenaline *in vivo* the tissue exports free fatty acids, whether this has any physiological significance is unknown (Ma and Foster, 1986; Himms-Hagan, 1989).

1.2G.ii. Lipoprotein Lipase.

Lipoprotein lipase (LPL) is present on the capillary endothelium in brown adipose tissue (Blanche-Mackie and Scow, 1983). Its action enables BAT to obtain fatty acids from dietary lipids (from circulating chylomicrons) and from fatty acid synthesised in the liver (from circulating VLDLs). Acute exposure to cold causes a large selective increase in BAT LPL activity and protein levels, which shows a slight lag period and initially exceeds tissue

growth (Carneheim *et al.*, 1984). The increase in LPL activity is preceded by elevation in LPL mRNA levels, both of which have been shown to be mediated by stimulation of the β -adrenergic receptors (Carneheim *et al.*, 1984; 1988, Mitchell *et al.*, 1989). Although cafeteria diet increases BAT activity it does not increase LPL activity (Bertin *et al.*, 1985; Carneheim *et al.*, 1988), except in the case of prior cold-acclimated rats (Bertin *et al.*, 1985).

Refeeding after fasting increases BAT LPL activity in an insulin-mediated response (Carneheim and Alexson, 1989). LPL activity is reduced in diabetes which, combined with the refeeding response, indicates a role for insulin, as well as noradrenaline, in the regulation of LPL activity in BAT. The apparent dual signal regulation has raised the possibility that either two LPL genes, or two promoters on the one gene may exist for BAT LPL (Mitchell *et al.*, 1988). There is a profound circadian rhythm in LPL activity in BAT which follows that seen in lipogenesis (Goubern and Portet, 1981; 1986).

LPL's role in supply of substrate to BAT for thermogenesis (and for esterification to replace lost triacylglycerol stores and produce essential phospholipids) makes this enzyme and its regulation highly important in BAT.

1.2G.iii. Mitochondrial Fatty Acid Oxidation.

Both the carnitine-dependant acyl-transport system and fatty acid β -oxidation cycle have been clearly demonstrated in BAT mitochondria (Flatmark and Pederson, 1975). BAT is also able to metabolise acetoacetate but not β -hydroxybutyrate (Agius and Williamson, 1980; Cooney *et al.*, 1986; Williamson and Ilic, 1985). A vital observation is that noradrenaline or fatty acid-stimulated respiration is totally dependant upon mitochondrial fatty acid oxidation (Bukowiecki, 1984). There is no substrate preference between long-chain fatty acids; all are equally able to stimulate, and be a fuel for, mitochondrial oxidation (Bukowiecki, 1984; Bukowiecki *et al.*, 1981). During intense thermogenesis, such as arousal from hibernation, all fatty acids present in the bulk lipid stores in BAT are utilised in mitochondrial oxidation (Carneheim *et al.*, 1989).

BAT mitochondria also contain three broad specificity acyl-CoA hydrolases for

short-, medium- and long-chain acyl-CoAs (Alexson and Nedergaard, 1988; Berge *et. al.*, 1979; Normann and Flatmark, 1984). There appears to be some species variation in hydrolase activities, but all three activities are elevated in mitochondria of cold-acclimated hamsters (Alexson and Nedergaard, 1988).

1.2G.iv. Peroxisomal Fatty Acid Oxidation.

The concentration of peroxisomes in BAT is elevated during cold-stress (Cannon *et. al.* 1982; Nedergaard *et. al.* 1980; Seydoux *et. al.*, 1986).

The role of peroxisomal fatty acid oxidation may be to selectively shorten very-long chain fatty acids, which are not good substrates for mitochondrial oxidation, resulting in production of fatty acids which are utilised by the mitochondria. The extent of oxidation in the peroxisomes is only minor in relation to the overall level (Alexson and Cannon, 1984; Nedergaard *et. al.*, 1980; Normann and Flatmark, 1982).

Other possible roles for peroxisomal fatty acid oxidation are protection against peroxidation of unsaturated fatty acids, via the high levels of catalase present (Cannon *et. al.*, 1982), or provision of substrates for stimulated lipogenesis in the cytosol (Himms-Hagan, 1986).

1.2G.v. Lipogenesis in Brown Adipose Tissue.

Research relating to lipogenesis (fatty acid synthesis) in BAT shows a high degree of contradiction and major inconsistencies, depending upon which system was used. For example *in vivo*, stimulated lipogenesis clearly accompanies stimulated thermogenesis (*i.e.* oxidation) under most, but not all, conditions (Buckley and Rath, 1987; Gouben and Portet, 1986; Himms-Hagan, 1986; Trayhurn 1979; 1981). However *in vitro*, direct administration of noradrenaline or fatty acids is inhibitory for lipogenesis (Himms-Hagan, 1989). In addition it is known that in the liver, oxidation and synthesis of fatty acid is usually mutually exclusive, due to the regulation of carnitine palmitoyl transferase by malonyl-CoA, an intermediate in lipogenesis (Saggerson and Carpenter, 1982). It is possible that this may also be the case in BAT, although there is no supportive evidence to date. Possible explanations of the differences between *in vivo* and *in vitro* results could be

due to differences between the two experimental approaches employed. Also in some cases pharmacological concentrations of noradrenaline were used, which have been shown to have different effects on BAT from the physiological levels normally used (see section 1.2G.i.), in addition there are physiological considerations such as the time of day and composition of diet (high fat diets reduce lipogenesis in BAT) (Mercer and Trayhurn, 1984; Rothwell *et al.*, 1983; Storlien *et al.*, 1986).

As fatty acids play such a vital role in thermogenesis, as an intracellular signal and a fuel, it would be expected that their levels would be highly regulated, with a continued supply available to the tissue. The immediate provision of fatty acids is via noradrenaline stimulation of HSL activity. HSL degrades intracellular stores of triglyceride and subsequent activation of LPL provides fatty acids from circulating triglycerides. Only one method of reduction of fatty acid levels in the cytosol exists in BAT, namely conversion to the acyl-CoA ester, catalysed by fatty acyl-CoA synthetase (FAS). This enzyme has a high activity in BAT (Flatmark and Pederson, 1975) and is predominantly localised on the outer mitochondrial membrane (Baht and Saggerson, 1988a; Pederson *et al.*, 1975). It is unlikely that FAS is the rate-limiting enzyme as it provides the substrate for both oxidation and esterification of fatty acids. Acyl-CoA levels in BAT remain constant until 8-9 days into cold-acclimation (Normann and Flatmark, 1984; Donatello *et al.*, 1988)

There is a BAT specific increase in lipogenesis during hypothyroidism (Baht and Saggerson, 1988b).

It is likely that noradrenaline inhibits lipogenesis in BAT *in vivo* (Agius and Williamson, 1980; Gibbins *et al.*, 1985). Addition of fatty acid to isolated adipocytes inhibits lipogenesis. This is perhaps unsurprising as addition of product generally leads to reduction in production of that product (Baht and Saggerson, 1988b; Saggerson *et al.*, 1988). The regulation point in lipogenesis may lie with the enzyme acetyl-CoA carboxylase, which is inhibited by noradrenaline (McCormack *et al.*, 1986; Gibbins *et al.*, 1985), with the large increases in intracellular fatty acid levels, induced by stimulation of the tissue, being a large factor. The capacity for lipogenesis in BAT is equal to that for

fatty acid oxidation (Nicholls *et. al.*, 1986; Nicholls and Locke, 1984).

1.2H. Glycerolipid Synthesis in Brown Adipose Tissue.

Research into glycerolipid synthesis in BAT has been sparse, but recent reports have studied the central enzymes involved in the production of phospholipids and triglyceride (Baht and Saggerson, 1988a, ; 1989; Darnley and Saggerson, 1988; Saggerson *et. al.*, 1988; Mercer and Williamson, 1988; Mitchell and Saggerson, 1991 which reports work also presented here).

In contrast to lipogenesis, glycerolipid synthesis is not inhibited by fatty acids (Saggerson *et. al.*, 1988), and is increased *in vivo*, with associated lipolysis and thermogenesis, by noradrenaline or cold stress (Brooks *et. al.*, 1983; Mercer and Williamson, 1988), and *in vitro* by noradrenaline (Baht and Saggerson, 1989; Ebner *et. al.*, 1987). Fatty acid esterification is inhibited by fasting and rapidly restored by refeeding, a response mediated by the sympathetic nervous system but requiring the presence of insulin (Mercer and Williamson, 1988). High fat diets inhibit BAT fatty acid synthesis and esterification from glucose and prevents insulin stimulation of these processes (Storlien *et. al.*, 1986).

Baht and Saggerson (1988a) showed that the central glycerolipid synthesis enzymes in BAT, excluding FAS, are predominantly microsomal but glycerolphosphate acyltransferase (GPAT) also showed mitochondrial activity. The same authors estimated that the capacity for fatty acid esterification in BAT is equal to the capacity for oxidation of fatty acids.

The effect of cold stress on the central enzymes of glycerolipid synthesis in BAT has been studied by Darnley *et. al.* (1988). They report that mitochondrial GPAT (but not microsomal GPAT), Mg²⁺-dependant phosphatidate phosphohydrolase (PPH) and mono-acyl-glycerolphosphate acyltransferase (MGPAT) activities are all elevated after only 24h. of cold stress and continue to rise for up to 12 days. No change in FAS activity was reported. The increase in mitochondrial GPAT and PPH activities was large (3-5 fold at 3 days cold exposure). This phenomenon alone merits further investigation of these enzymes

in cold-stressed BAT. It should be noted that, at the time at which the dramatic increase in the two key enzymes in the glycerolipid synthesis pathway occurs (approximately 3 days cold exposure), the tissue is beginning to exhibit the extensive changes seen during cold acclimation. At 3 days cold exposure total tissue protein and total tissue DNA levels are significantly elevated (Darnley *et. al.*, 1988). The adaptations seen in BAT, at and beyond 3 days cold-exposure, include large increases in mitochondriogenesis and cellular proliferation (requiring the production of phospholipids for membrane production), and initiation of replenishment of triglyceride stores, depleted during the first 24h. of cold stress. All the above changes would require increased utilisation of the glycerolipid synthesis pathway. It is also important to realize that any increase in fatty acid esterification will, in theory, reduce the available substrate for thermogenesis. Therefore, a fine balance between the two processes of maintaining thermogenesis and allowing tissue growth must be struck. All the above considerations make the elucidation of the regulation of the glycerolipid synthesis pathway interesting especially in terms of triglyceride replacement and early events in tissue hypertrophy in chronically stimulated BAT.

1.3. Aims of the Study.

- 1) To confirm the effect of cold-exposure on BAT and the glycerolipid synthesis pathway enzyme activities in the tissue reported by Darnley *et. al.* (1988).
- 2) To investigate the adrenergic signals involved in the regulation of activities of the glycerolipid synthesis enzymes in BAT. The extent of the β -adrenergic pathway involvement in regulation of the activity of the enzymes in the glycerolipid synthesis pathway was investigated using a novel BAT specific β_3 -adrenergic agonist, BRL 26830A. The effect of BRL 26830A on the glycerolipid synthesis enzyme activities in BAT, over a three day period, both in the warm (21°C) and in the cold (4°C) was investigated. Phenylephrine was used to investigate the involvement of the α -adrenergic pathway in regulation of the enzymes in the glycerolipid synthesis pathway.
- 3) To investigate the effect of hormonal status on the regulation of the activity of

mitochondrial GPAT and Mg^{2+} -dependant PPH in warm-adapted and cold-exposed BAT. The effects of diabetes, hypothyroidism, hyperthyroidism and adrenalectomy were investigated.

4) To investigate whether cold-induced changes in the levels of activity of mitochondrial GPAT and Mg^{2+} -dependant PPH in BAT are dependant upon increased gene expression, increased translation or activation of an intracellular pool of enzyme.

5) To investigate whether the activity of liver mitochondrial GPAT is confined to the outer mitochondrial membrane or is present in both inner and outer membranes. Normal, starved, and diabetic states were investigated.

6) To purify and characterize the mitochondrial form of GPAT from rat liver.

MATERIALS AND METHODS.

CHAPTER TWO.

2.1. MATERIALS.

2.1A. Commercial Preparations.

Routinely used reagents were of the highest possible grade and were obtained from BDH Ltd., Poole, Dorset, U.K. and May and Baker Ltd., Dagenham, Essex, U.K.

The following were supplied by:-

Amersham International, Amersham, Bucks., U.K.; [^{14}C]-glycerol-3-phosphate, [^{14}C]sucrose and [^3H]guanosine di-phosphate.

Fisons PLC, Loughborough, U.K.; ammonium sulphate, hydrochloric acid.

Koch-Light, Haverhill, Suffolk, U.K.; polyethylene glycol.

Biorad Ltd., Hemel Hempstead, Herts., U.K.; Biobeads[®].

Calbiochem Corporation, Novabiochem, Nottingham, U.K.; Calbiosorb[®].

Boehringer Mannheim (U.K.), Lewes, Sussex, U.K.; hexokinase, glycerol-3-phosphate, glucose-6-phosphate dehydrogenase, ^{reduced}nicotinamide-adenine di-nucleotide phosphate (NADPH), ^{reduced}nicotinamide-adenine di-nucleotide (NADH), guanosine di-phosphate, palmitic acid, palmitoyl-CoA.

Sigma Chemical Company, Poole, Dorset, U.K.; deoxyribonucleic acid (DNA), diamino benzoic acid (DABA), phosphatidylcholine (from egg yolk), adenosine tri-phosphate (ATP), oxidised nicotinamide adenine di-nucleotide phosphate (NADP), Triton WR 1339 (Tyloxapol), 1,N⁶-etheno-CoA, oleoyl-CoA, N-ethylmaleimide (NEM), *rac*-glycerophosphate, 5-5' dithiobis(2-)nitrobenzoic acid (DTNB), 1-palmitoyl-*sn*-glycerol-3-phosphate, ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA), ascorbic acid, 6-n-propyl-2-thio-uracil (PTU), tri-iodothyronine (T₃), fatty acid-poor albumin, streptozotocin, phenylephrine, cycloheximide, actinomycin D, Tris-base, ethylene diamine tetra-acetic acid (EDTA), cholic acid (sodium salt), Triton X-100, cytochrome c, rotenone.

Camlab, Macherey Nagel and Co., Duren, Germany: Polygram SIL G TLC plates.

Miles U.K. Ltd, Slough, Berks., U.K: Clinstix glucose reagent sticks.

BRL-26830A was a very kind gift from Dr. M. Cawthorne, Smith Kline Beecham, Welwyn, Herts., U.K.

2.1B. Laboratory Preparation of Sodium Phosphatidate.

Sodium phosphatidate was prepared from phosphatidylcholine essentially as described by Davidson and Lang (1958) and Ansell and Hawthorne (1964).

Phospholipase D was extracted from cabbage leaves (white cabbage preferably) by blending (Waring blender or similar) with water at a ratio of 2:1, cabbage : water.

The resulting homogenate was passed through cheese cloth before centrifugation at 4°C for 30 min., at 13 000g_{av} (Sorvall RC-5B). The resultant supernatant was then used as the source of phospholipase D. After buffering to pH 5.6 with sodium acetate (0.1 M final concentration) and addition of calcium chloride (0.1 M final concentration) the extract was added to 30 ml of diethyl-ether, containing 0.5-1.0 g of phosphatidylcholine (from egg yolk), to give a final volume of 90 ml. The mixture was shaken vigorously and incubated at 26°C for 3 h. with frequent shaking. The mixture was then left overnight at room temperature.

Extraction of the required phosphatidate from the two phase mixture was carried out as follows:-

The mixture was acidified to pH 3.0 using glacial acetic acid and the ether layer decanted off and stored. The aqueous phase was then extracted four times with 30 ml of diethyl-ether and the ether phases pooled (final volume approximately 150 ml). The ether was washed twice with 40 ml of water saturated with EDTA, then dried by the addition of anhydrous sodium sulphate in excess. The ether was then evaporated to dryness under a stream of oxygen-free nitrogen and the residue redissolved in 25 ml of ethanol:diethyl-ether (4:1 v/v). After cooling to 5°C the resultant precipitate was removed by centrifugation in glass tubes (MSE Bench centrifuge). The supernatant was recooled to 0-4°C and 5 ml of 0.5 M alcoholic sodium hydroxide was added drop-wise with constant stirring to precipitate the phosphatidate. The phosphatidate was collected by centrifugation and

washed once with ethanol:diethyl-ether and once with absolute ethanol. The remaining precipitate was redissolved in 10 ml of diethyl-ether and any remaining particles removed by centrifugation. Finally 20 ml of acetone was added to the solution, at 40°C, and the precipitated phosphatidate washed several times with acetone, dried under oxygen-free nitrogen and stored at -20°C.

Yield was approximately 70-75% of the starting weight of phosphatidylcholine by weight.

2.1B. Assessment of Purity of Phosphatidate.

The purity of prepared phosphatidate was assessed using thin layer chromatography (TLC) on Polygram SIL G TLC plates (20 cm x 20 cm) with 0.25 mm silica gel plated on plastic sheets. The solvent system used was chloroform: methanol: formic acid: water (65: 26: 7: 3.5).

A Whatman No. 1 paper-lined TLC tank was allowed to equilibrate overnight at room temperature with 200 ml of solvent. Phosphatidylcholine and commercially prepared phosphatidate were used as standards and loaded along side the laboratory-prepared phosphatidate. All samples were dissolved in chloroform, spotted onto TLC plates and air-dried.

The plate was placed in the pre-equilibrated tank and allowed to develop until the solvent front had reached 2-3 cm from the top of the plate. The plate was then air-dried and sprayed with Zinzadze reagent for phospholipids. Zinzadze reagent was prepared in the following manner:- 6.85 g sodium molybdate and 0.4 g hydrazine sulphate were dissolved in 250 ml of water and 250 ml of concentrated sulphuric acid was added very slowly and with great care. When the solution was cool it was made up to 1 l with water. The reagent reacts with phospholipids giving a blue colour. A single spot in the laboratory-prepared phosphatidate track was seen, corresponding to that seen with the commercial phosphatidate on each occasion purity was checked.

2.2 ANIMALS.

All animals used in this study were male Sprague-Dawley rats aged approximately 6

weeks (180-200 g), except those used to study the effects of hypothyroidism (see below). Animals were maintained on a 13 h.-light / 11 h.-dark cycle, with light from 06:00 to 19:00 h. Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex.) was available to the animals *ad libitum* and contained (w/w) 21% digestible protein, 4% digestible crude oil and 39% starches and sugars.

Hypothyroidism was induced by maintenance on an iodine-deficient diet (Special Diet Services, Witham, Essex) and treatment with 6-n-propyl-2-thiouracil (PTU) in the drinking water (final concentrations; 0.01% w/v PTU and 0.3% v/v ethanol) for at least four weeks prior to further treatments. These animals were age-matched to euthyroid controls and all animals were approximately 9 weeks old at time of death.

Hyperthyroidism was induced by daily subcutaneous injection of tri-iodothyronine (T_3). Injections were performed (0.2 ml of 1mg/kg body weight T_3 in 10 mM sodium hydroxide, containing fatty acid-poor albumin (0.03% w/v)) at 10:00h on each of four consecutive days and the rats sacrificed at the same time 24 h. after the fourth injection.

Diabetes was induced 3 days before further treatment(s) by subcutaneous injection of streptozotocin (80 mg/kg body weight) dissolved in 0.2 ml of 50 mM sodium citrate buffer (pH 4.0) containing 0.15 M sodium chloride. All animals taken as diabetic showed a strong `Clistix` test for urinary glucose (>0.5% w/v). Also all animals in the diabetic and appropriate control groups had blood glucose levels measured to confirm induction of diabetes.

Adrenalectomized and sham-adrenalectomized animals were purchased from Charles River and kept as described above for at least 5 days before further experimental treatments. The adrenalectomized animals were maintained on drinking water containing 0.9% sodium chloride (w/v, final concentration) to maintain the animals' salt balance.

Phenylephrine (0.7 mg/kg body weight) and BRL-26830A (1 mg/kg body weight) were administered by interperitoneal injection (in 0.2 ml of 0.15 M sodium chloride), on 3 consecutive days at 10:00 h. The animals were sacrificed 24 h. after the final injection. Cycloheximide (2 mg/kg body weight) and Actinomycin D (0.5 mg/kg body weight) were

injected subcutaneously (in 0.2 ml of 0.15 M sodium chloride), at 10:00 h. immediately prior to any further experimental treatment.

All animals were kept at 21°C, except those exposed to cold, which was at 4°C for 3 days (those animals which were made hypothyroid were exposed to cold for 2 days only as longer periods were not tolerated).

2.3 Tissue Preparations.

2.3A. Brown Adipose Tissue.

Animals were sacrificed by cervical dislocation and the interscapular brown adipose tissue excised and placed in ice-cold homogenisation medium. The medium contained 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol (DTT) and 1 mM EDTA. Each animal's interscapular brown adipose tissue (IBAT) was weighed and then homogenised separately in 7 ml of homogenisation medium using an Ultraturrx homogeniser for approximately 20-30 sec. The samples were then left on ice for at least 5 min. before centrifugation at 3 000g (MSE bench centrifuge) for 5 min. The defatted tissue samples were aspirated away from the floating fat and aliquoted into 1 ml fractions for storage at -70°C. Prior to storage 50 µl of each sample was added to 50 µl of 50% trichloroacetic acid (TCA) and left on ice for a minimum of 30 min. These samples were used to determine deoxyribonucleic acid levels (see below) and could be stored at -20°C until required.

An alternative preparation was used to provide IBAT mitochondria for GDP binding studies. Pooled IBATs from two normal or treated animals were homogenised in the medium described above with the addition of fatty acid-poor albumin to a final concentration of 1% (w/v). Homogenisation was carried out using a Potter-Elvehjem homogeniser fitted with a motor-driven teflon pestle rotating at 500 rev./min. with a radial clearance of 0.19 mm.

Mitochondria were obtained from the homogenate by the method of Saggerson and Carpenter (1982). The homogenates were spun at 10 000 rpm for 10 min. at 4°C (Sorvall RC-5B) to remove the mitochondria from floating fat and microsomal plus cytosolic

fractions. The pellet from the above spin was resuspended and recentrifuged at 2 300 rpm (10 min., 4°C) to remove cell debris and nuclei. The supernatant was centrifuged at 10 000 rpm for 10 min. at 4°C, to pellet the mitochondria. The mitochondrial pellet was washed several⁽²⁻³⁾ times to remove any residual contamination and finally resuspended in homogenisation medium minus the DTT and the fatty acid-poor albumin.

2.3B.i. Liver.

For studies on the Liver mitochondrial glycerolphosphate acyltransferase (GPAT), liver mitochondrial membranes were prepared as described by Ghadiminejad and Saggerson (1990).

Rats were sacrificed as described above, the liver excised and placed in ice-cold buffer-A (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and 0.1 mg/ml PMSF). The livers were then chopped and washed with buffer-A to remove the majority of blood associated with the tissue. Buffer-A was added to give approximately 50 ml buffer/ liver. The chopped liver was then homogenised using a homogeniser identical to that used in the preparation of mitochondria in the binding studies. The homogenate was then centrifuged at 0-4°C for 10 min. at 600g in a Sorvall RC-5B centrifuge. The supernatant was decanted and put to one side on ice. The pellet was resuspended in buffer-A to give a final volume close to that prior to homogenisation, and rehomogenised to improve mitochondrial yield by ensuring most of the liver cells were broken thus releasing mitochondria. The second homogenate was centrifuged in an identical manner to that of the first homogenate. The two supernatants were combined and centrifuged at 9 200g for 15 min. at 0-4°C. The pellet from this spin yields the mitochondrial fraction, the mitochondria were washed twice with buffer-A and finally resuspended in buffer-B (20 mM potassium phosphate buffer, pH 7.4, and 0.1 mg/ml PMSF). The post-mitochondrial supernatant was either discarded or centrifuged at 105 000g, for 1h. at 4°C (70.1 Ti rotor, Beckman L8 High-Speed centrifuge) to yield a microsomal fraction and a cytosolic fraction. The microsomal fraction obtained was used for comparison between the mitochondrial and

* The probe used had a 9.5 mm tip diameter and the machine operated at 23 KHz

65

microsomal GPAT in different states and conditions.

Membranes were produced from the intact mitochondrial fraction by sonication (Branson Sonic Co., W350 probe sonicator), for 15 sec. periods with 10 sec. intervals between sonications, on ice, at maximum ^{(14 micron) *} amplitude. Up to 10 sonication periods were used per membrane preparation. The membranes were separated from the matrix and inter-membrane space by centrifugation at 105 000g for 40 min. at 0-4°C. The membrane pellet was resuspended in a minimum amount of buffer-B and stored at -70°C, with no discernable loss of GPAT activity over an extended period (up to 1 yr.).

2.3B.ii. Preparation of Inner and Outer Mitochondrial Membranes.

Inner and outer mitochondrial membranes were prepared by the method of Ghadiminejad and Saggerson, (1990).

Liver mitochondria were prepared as described above, except that final resuspension of the mitochondrial pellet was into buffer-B minus PMSF but with fatty acid-poor albumin added (0.2 mg/ml). After 20 min. swelling of the mitochondria, on ice, ATP (dissolved in 20 mM potassium phosphate, pH 7.2) and MgCl₂ were added to give final concentrations of 1 mM. After 5 min. on ice to allow shrinkage of the mitoplasts, the suspension was centrifuged at 35 000g_{av} for 20 min.. The resulting pellet was resuspended in modified buffer-B and most of the inner membrane (as mitoplasts) removed by centrifugation at 19 000g_{av} for 15 min.. The supernatant was further centrifuged at 35 000g_{av} for 20 min. The pellet from this second spin was resuspended in 20 mM potassium phosphate, pH 7.2 to give a crude outer membrane fraction. This outer membrane fraction was then applied to the top of a triple-layered sucrose density gradient, consisting of; 1.2 ml of 0.74 M ($\rho = 1.094$), 2.4 ml of 1.1 M ($\rho = 1.142$) and 2.4 ml of 1.5 M ($\rho = 1.192$). After centrifugation for 1 h. at 110 000g_{av} the band at the 0.74 M/1.1 M interface was recovered, diluted at least 1 in 4 with 20 mM potassium phosphate, pH 7.2, and recentrifuged at 115 000g_{av} for 1 h.. The resulting pellet was resuspended in 250 mM sucrose-medium containing 5 mM potassium phosphate, pH 7.2, and stored at -70°C until

required.

The pellet below the 1.5 M sucrose layer was combined with the mitoplast fraction from above, resuspended in buffer-A minus PMSF and sonicated as described for total membrane production above. The sonicated suspension was centrifuged at 115 000g_{av} for 1 h. and the inner membrane pellet washed once with modified buffer-A. The inner membranes produced in this way were stored in modified buffer-A at -70°C until required. Purity of the two membrane fractions was assessed using specific membrane-marker enzymes (see Results section).

2.3C.i. Solubilisation of Liver Mitochondrial Membrane Proteins.

Two methods for solubilisation of total mitochondrial membranes (*i.e.* both inner and outer membranes) to yield GPAT in the soluble fraction were employed;-

A) Membranes were suspended in 10 mM Tris-HCl, pH 8.0, 1 M KCl or 1 M NaCl, to give a protein concentration of approximately 10 mg/ml. The suspension was brought to 0.5% cholate (sodium salt) (w/v) with constant mixing. After 10 min. on ice the remaining membrane fragments were sedimented away from the solubilised protein by centrifugation at 105 000g, for 30 mins at 0-4°C. A 70% recovery of initial GPAT activity was routinely observed in the soluble fraction. GPAT activity was stabilised in the soluble state by immediate removal of the detergent (mixing with Calbiosorb® or Biobeads® for at least 1h. on ice) and taking the solution to 20% glycerol (v/v). This method is adapted from that described by Monroy *et. al.* (1973).

B) Membranes were suspended in 20 mM glycine-NaOH buffer, pH 7.5, 2 mM dithiothreitol (DTT), to give a protein concentration of approximately 5-10 mg/ml. The suspension was brought to 0.5% Triton X-100 (v/v) and sonicated (Branson Probe sonicator, maximum amplitude*) for 30 sec. on ice. The sonication was repeated twice within a 1 h. period, after which time the suspension was left on ice for a further 2 h. Membrane fragments not solubilised by this time were removed by centrifugation at 165 000g for 90 min., at 4°C. Routinely a 50% recovery of initial GPAT activity was obtained.

GPAT activity was stabilised in the soluble state by removal of the detergent (as in A) and bringing the solution to 20% glycerol (v/v). The buffer described above was later found not to be essential, solubilisation being the same in any of the buffers tried at pH 7.5. This method is adapted from that described by Mok and McMurray (1990).

The soluble GPAT activity obtained by the above methods proved to be stable for at least 1 year when stored at -70°C providing excess freeze-thawing was avoided.

2.3C.ii. Ammonium Sulphate Precipitation.

Saturated ammonium sulphate was added to a set volume of soluble extract (from the liver mitochondrial membrane solubilisation, after detergent removal) to give an accurate final percentage of ammonium sulphate in the mixture. Addition of the saturated salt solution was drop-wise, with constant stirring, on ice. The mixture was left stirring on ice for 30 mins. to ensure precipitation was complete. The precipitated material was sedimented by centrifugation (16 000g, 30 mins. at 4°C) using a Sorvall RC-5B centrifuge. GPAT activity was assayed in the supernatant and in the resuspended pellet as described in Enzyme Assays. A range of 0-50% ammonium sulphate (final concentration), with 5% increments, was studied.

2.3C.iii. Polyethylene Glycol Precipitation.

50% polyethylene glycol solution was added to a set volume of soluble extract (see above) to give an accurate final percentage of polyethylene glycol in the mixture. The polyethylene glycol solution was added to the soluble extract slowly, with constant stirring, on ice. The mixture was left stirring on ice for 30 mins. to allow complete precipitation to occur. The precipitated material was sedimented by centrifugation (see ammonium sulphate precipitation for conditions). GPAT activity was measured in the supernatant and resuspended pellet (for assay see Enzyme Assays section). A range of 0-30% polyethylene glycol (final concentration), with 3% increments, was studied.

2.3C.iv. Ion-Exchange Chromatography.

Ion-exchange chromatography was performed on a fast protein liquid chromatography (FPLC) system (Pharmacia LKB, Sweden).

Soluble extract from cholate solubilisation of liver mitochondrial membranes (using a 20 mM potassium phosphate buffer, pH 7.4) was de-salted, after detergent removal, by dilution and re-concentration using an Amicon ultrafiltration unit and a YM 30 filter (allows only materials of less than 30 kDa to pass through).

The de-salted soluble extract (or Triton solubilised soluble extract) was centrifuged at 105 000g for 1h. at 4°C (Beckmann L-8 High-speed centrifuge) to remove any insoluble material. The supernatant was filtered through a 0.22 µm filter and 2 ml aliquots applied to the chromatographic column.

The ^{1 ml} column, either Mono Q (anionic exchange), or Mono S (cationic exchange), was pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.4, 20% glycerol (buffer A). The 2 ml aliquot was applied to the column at a flow rate of 0.5 ml/min. After unbound material had washed through the column (4-10 column volumes of buffer A), bound material was eluted with a linear gradient of 0-600 mM NaCl in buffer A over 20 column volumes. One ml fractions were collected and assayed for GPAT activity. The above procedure was repeated at various pHs.

2.3C.v. Hydrophobic Interaction Chromatography.

Hydrophobic interaction chromatography was performed on a FPLC system.

Soluble extract from cholate or Triton solubilisation of mitochondrial membranes was pre-treated as for ion-exchange chromatography except that the extract was not de-salted (Triton solubilised soluble extract was made up to 1 M NaCl prior to centrifugation).

The hydrophobic interaction column used was the ^{1 ml} Phenyl-superose (Pharmacia LKB, Sweden) variety and was pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 1 M NaCl (buffer B). 2 ml of soluble extract (in buffer B) was applied to the column at a flow rate of 0.25 ml/min. Once the unbound material had passed through the column (6-10 column volumes), bound material was eluted with a linear gradient of 1-0 M NaCl in buffer B over 15 column volumes. 1 ml fractions were collected and assayed for GPAT activity.

2.3C.vi. Affinity Chromatography.

Soluble extract from solubilisation of liver mitochondrial membranes was pre-treated as for ion-exchange chromatography.

0.5 ml aliquots of extract were mixed with 0.5 ml of affinity matrix (either blue-sepharose or palmitoyl-CoA agarose) in an eppendorf tube for 30 min. on ice. The matrix was sedimented by centrifugation in a bench microfuge (2 min., 2 800g) and the supernatant removed and assayed for GPAT activity. Material bound to the matrix was eluted by washing with 0.5 ml of buffer B (see hydrophobic interaction chromatography section) and resedimented. The eluted supernatant was also assayed for GPAT activity.

2.3D. Protein Estimation.

Protein in each sample was estimated using the method of Lowry *et. al.* (1951).

50 μ l of sample was added to 150 μ l of 0.1 M sodium hydroxide (NaOH) and mixed using a Whirlie-mix vortex, The 200 μ l was then added to 2 ml of solution A (consisting of 2% sodium carbonate in 0.1 M NaOH, 5% potassium-sodium-tartrate and 1% copper sulphate in a 100:1:1 ratio respectively) which had been freshly prepared. After mixing, 50 μ l of neat Folin-Ciocalteu's reagent was added and the solution mixed well. The solutions were left for 30-60 min., to allow full colour development, before measuring the absorbance at 660 nm. A standard curve of 0-100 μ g of protein was constructed (1 mg/ml fatty acid-poor albumin stock used) for each new solution A. The protein content of each sample was determined from the standard curve.

2.3E. DNA Estimation.

DNA was estimated in each sample using the of Switzer and Summer (1971). As stated above, 50 μ l of fresh sample was added to 50 μ l of 50% trichloroacetic acid and this could either be stored at -20°C or the assay continued. If frozen, samples were thawed at room temperature and centrifuged at 2 800gav (microfuge) for 2 min.. The supernatant was discarded and the tubes inverted to drain on tissue paper. 400 μ l of 10 mM potassium acetate in ethanol was added to each sample (to remove residual water) and the samples sonicated. The samples were centrifuged again and the supernatant discarded. The samples

were allowed to air dry overnight. The DNA standard curve was usually set up at this stage, ranging from 0 to 4 μg DNA.

On day 2, 20 μl of freshly prepared diamino-benzoic acid (DABA) was added to each sample (including the standards). All samples were mixed and sonicated before incubation at 60°C for 30 min.. After cooling, 0.58 ml of 0.6 M perchloric acid was added to each sample and the fluorescence at excitation 420 nm and emission 520 nm was measured using a Perkin-Elmer PE 3000 fluorescence spectrophotometer.

2.3F. Glucose Assay.

Glucose in the serum of diabetic animals and their controls was measured using an adaptation of the method of Slein (1963).

Blood was taken by cardiac puncture using a heparinised syringe immediately after death. Plasma was obtained by centrifugation for 5 min. at 2 800g (Eppendorf 5412 microfuge), diluted 1 in 10 and then used to estimate blood glucose levels. 20 μl of the 1/10 serum was added to 2 ml of freshly prepared assay buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl_2 , 0.5 mM ATP, 0.2 mM NADP and 0.2 mg/ml fatty acid-poor albumin). 0.95 ml of double distilled water and 20 μl (of a 1/5 dilution of the commercial stock) glucose-6-phosphate dehydrogenase was added to the assay mixture and the absorbance at 340 nm taken as the zero value. 10 μl of hexokinase (1/5 dilution of commercial stock) was added and after 5 min. the absorbance at 340 nm was noted. Using the extinction coefficient of $6.22 \times 10^3 \text{ mol}^{-1} \text{ ml}$ the amount of glucose in each sample was determined.

2.4. ENZYME ASSAYS.

2.4A. Fatty acyl-CoA Synthetase.

ATP-dependant long-chain fatty acyl-CoA synthetase (FAS) (E.C.6.2.1.3.) was assayed fluorometrically by the method of Hall and Saggerson (1985).

The assay buffer consisted of 0.35 M Tris-HCl, pH 7.4, 8 mM MgCl_2 , 5 mM DTT, 1 mg/ml Triton WR 1339, 5 mM ATP, 60 μM palmitic acid and 0.5 mM 1,N⁶-etheno-CoA.

The assay was initiated by the addition of 10 μ l of sample to 0.2 ml of assay buffer. After 7 min. at 25°C the assay was terminated by addition of 2 ml of fatty acid-poor albumin (6.45 mg/ml) and 2 ml of ice-cold 0.3 M TCA. The assays were left on ice for 5 min. and then centrifuged at 200g for 5 min. (MSE Minor bench centrifuge). The supernatant was discarded and the pellet washed 2-3 times with 0.3 M TCA. Finally the pellet was redissolved in 3 ml of 25 mM NaOH and the fluorescence of the samples determined at excitation and emission wavelengths of 275 nm and 410 nm respectively (Perkin-Elmer PE 3000 fluorescent spectrophotometer). A linear standard curve of 0-15 nmol of etheno-CoA dissolved in 25 mM NaOH was constructed and used to calculate enzyme activity. Enzyme activity was expressed as nmol/min/100 μ g DNA (or/mg protein).

2.4B. Glycerolphosphate Acyltransferase.

Glycerolphosphate acyltransferase (E.C.2.3.1.15.) (GPAT) in brown adipose tissue was assayed radiochemically by the method of Rider and Saggerson (1983) and Saggerson *et. al.* (1980).

The assay medium contained 100 mM Tris-HCl, pH 7.4, 0.7 mM DTT, 1 mM [U-¹⁴C-]glycerol-3-phosphate (0.5 μ Ci/assay) and 1.75 mg/ml fatty acid-poor albumin. For mitochondrial GPAT, 40 μ M palmitoyl-CoA and 10 mM N-ethylmaleimide (NEM) were also present; for microsomal GPAT 120 μ M oleoyl-CoA was used. These separate substrates and the thiol-reagent NEM are used to clearly distinguish the two GPAT activities in a homogenate (Bates and Saggerson, 1979; Saggerson *et. al.*, 1980). Variations on the above conditions are listed where appropriate.

The assay was initiated by addition of 0.1 ml of tissue sample to 0.9 ml of the above buffer (palmitoyl-CoA, oleoyl-CoA and [U¹⁴C-]glycerol-3-phosphate were added to the assay buffer from stock solutions). After 8 min. at 30°C the assay was terminated by addition of 2 ml of butan-1-ol saturated with 0.1 M HCl. After mixing and centrifugation at 2 800g (MSE bench centrifuge) the top butanol layer (containing the butanol-soluble reaction products) was harvested and washed twice with 10 mM *rac*-glycerolphosphate in

Figure 2.1. Mitochondrial GPAT Time Course

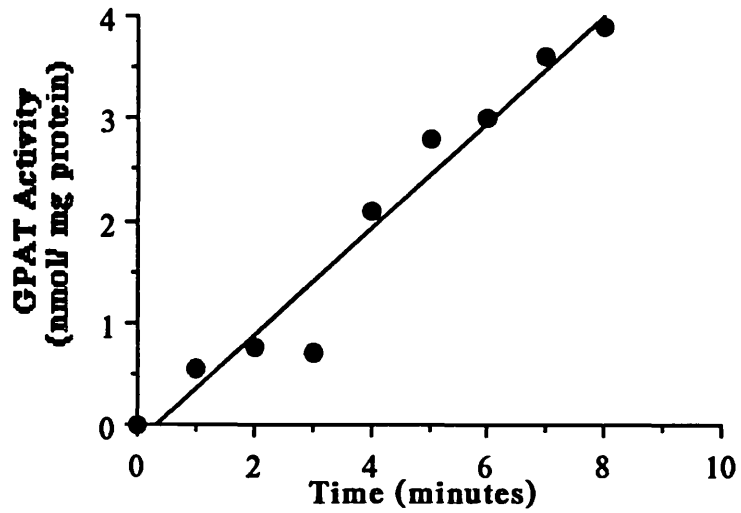


Figure 2.1. Figure 2.1 shows the change in substrate of mitochondrial GPAT over time under the assay conditions stated in the Glycerolphosphate Acyltransferase section. The graph illustrates A linear assay over time up to 10 mins.

0.1 M HCl saturated with butan-1-ol. 1 ml of the remaining butanol layer was added to 10 ml of Ecoscint A® for scintillation counting to determine the butanol soluble [¹⁴C]radioactivity and hence the GPAT activity. Enzyme activity was expressed as nmol/min/100µg DNA (or /mg protein) and the assay was found to be linear for up to 10 min. (see fig. 2.1).

For Liver GPAT activity measurements the following changes to the assay described above were made:- 6 mg/ml fatty acid-poor albumin was used instead of 1.75 mg/ml and 120 mM KCl was additionally present in the assay medium (Bates and Saggerson, 1979). Also the assay time was reduced from 8 to 5 min. as this gave more reproducible results especially after solubilisation of mitochondrial membranes.

2.4C. Monoacyl-glycerolphosphate acyltransferase.

Monoacyl-glycerolphosphate acyltransferase (E.C.2.3.1.51.) (MGPAT) was assayed spectrophotometrically by the method of Okuyama *et. al.* (1971) and Saggerson *et. al.* (1980).

The assay medium contained 50 mM Tris-HCl, pH 7.4, 120 mM KCl, 2 mg/ml fatty acid-poor albumin, 100 µM 5-5'-dithiobis(2-)nitrobenzoic acid (DTNB). 10 µl of 12 mM oleoyl-CoA was added to the medium (final concentration, 120 µM), mixed and 10-100 µl of sample added. After mixing a base-line at 412 nm absorbance was obtained. The reaction was initiated by addition of 40 µl of 33 mM Tris-HCl, pH 8.0, containing 65 nmol of 1-palmitoyl-sn-glycerol-3-phosphate (final assay volume was 1 ml). The reaction (transfer of CoA from oleoyl-CoA to DTNB) was followed at 412 nm, 25°C and the activity of MGPAT calculated using the extinction coefficient of 13.6 µmol⁻¹ ml. Enzyme activity was expressed as nmol/min/100µg DNA (or /mg protein). Linear absorbance traces were obtained for up to 5 min. (not shown) and generally a 3 min assay time was used.

2.4D. Phosphatidate Phosphohydrolase.

Phosphatidate phosphohydrolase (E.C.3.1.3.4.) (PPH) was assayed by the method of Taylor and Saggerson (1986). Inorganic phosphate release was measured as an indicator

Figure 2.2. PPH Assay Time Course

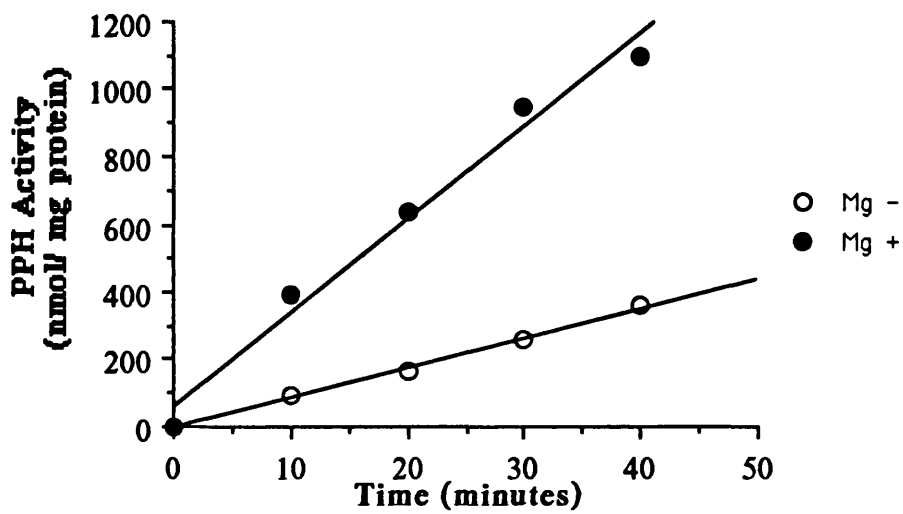


Figure 2.2. Figure 2.2 shows the change in PPH_{product} over time using the assay conditions stated in the Phosphatidate Phosphohydrolase section. The graph shows the assay to be linear with time for up to 40 mins.

of PPH activity.

The assay buffer contained 100 mM Tris-maleate buffer, pH 7.4, 1 mM DTT, 1 mM EGTA, 2.5 mM $MgCl_2$ (for Mg^{2+} -dependant activity only), 1 mM EDTA (for Mg^{2+} -independant activity only) and 0.75 mM sodium phosphatidate (prepared in the laboratory as described above). To 0.4 ml of the above assay medium, 0.1 ml of sample was added to initiate the reaction. After 20 min. at 37°C the reaction was terminated by the addition of 0.5 ml of ice-cold 10% TCA and the assays left on ice for a further 30 min.. Centrifugation at 2 800g followed the period on ice (2 min. in a Eppendorf 5412 microfuge) to remove any precipitation caused by addition of the TCA. Phosphate released into the medium was measured by the method of Ames and Dubin (1960). 0.5 ml of the medium was mixed with 1.5 ml of colour reagent (prepared fresh). The colour reagent consisted of 1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate in 0.5 M sulphuric acid. A standard curve of 0-100 nmol of inorganic phosphate was prepared for each new batch of colour reagent using a stock solution of 0.2 mM potassium dihydrogen orthophosphate in 100 mM Tris-maleate, pH 7.4, with an equal volume of 10% TCA. All samples were incubated at 45°C for 20 min. and then placed on ice to stop the colour-producing reaction. The absorbance at 820 nm was measured and the activity of Mg^{2+} -dependant PPH calculated from the standard curve produced and subtracting the Mg^{2+} -independant activity from that obtained in the presence of Mg^{2+} . Enzyme activity was expressed as nmol/min/100 μ g DNA (or /mg protein) and the assay was found to be linear for up to 40 min. (see fig. 2.2).

2.4E. NADP⁺ Cytochrome c-Reductase.

NADP⁺-cytochrome c-reductase (E.C.1.6.2.4.) was assayed spectrophotometrically by the method of Phillips and Langdon (1962).

In a 1 ml final volume, the assay contained; 0.33 M potassium phosphate buffer, pH 7.6, 1 mM potassium cyanide (KCN), 0.05 mM cytochrome c (added from a stock solution), 0.04 mM NADPH (added from a stock solution to initiate the reaction) and 10-80 μ l of sample. The change in absorbance at 550 nm, against a blank minus the NADPH,

was followed and enzyme activity calculated using an extinction coefficient of $18.5 \mu\text{mol}^{-1}\text{ml}$ and expressed as $\text{nmol}/\text{min}/100\mu\text{g DNA}$ (or $/\text{mg protein}$).

2.4F. Rotenone-insensitive NADH-Cytochrome *c*-Reductase.

Rotenone-insensitive cytochrome *c*-reductase (E.C.1.6.99.3.) was assayed spectrophotometrically by the method of Scottcasa *et. al.* (1967), Duncan (1966) and Booth and Clarke (1978).

The assay was carried out in a 1 ml final volume containing; 0.2 M potassium phosphate, pH 7.4, 1 mM KCN, 0.1% (w/v) cytochrome *c* and $5 \mu\text{M}$ rotenone. The rotenone was prepared by dissolving 5 mg in 2-3 ml of acetone, adding 100 ml of double distilled water and using $40 \mu\text{l}$ per 1 ml of assay medium. $50 \mu\text{g}$ of sample protein was added per 1 ml of assay medium (approximately $30 \mu\text{l}$). The assay was initiated by the addition of $20 \mu\text{l}$ of 3.7 mg/ml NADH (final concentration of 0.25 mM). The change in absorbance at 550 nm (against a blank of assay medium minus NADH) was followed and the enzyme activity calculated using the extinction coefficient of $18.5 \mu\text{mol}^{-1}\text{ml}$. Enzyme activity was expressed as $\text{nmol}/\text{min}/\text{mg protein}$.

2.4G. Succinate-Dependant Cytochrome *c*-Reductase.

Succinate-dependant cytochrome *c*-reductase was measured spectrophotometrically by the method of Scottcasa *et. al.* (1967).

The assay was carried out in a 3 ml final volume containing; 0.2 M potassium phosphate, pH 7.5, 1 mM KCN, 0.2% cytochrome *c* and 0.1 ml of sample. The assay was initiated by addition of 0.1 ml of 90 mM sodium succinate (final concentration of 3 mM). The reaction was followed at 550 nm (against a blank of assay medium minus sodium succinate) and enzyme activity calculated using the extinction coefficient $18.5 \mu\text{mol}^{-1}\text{ml}$. Enzyme activity was expressed as $\text{nmol}/\text{min}/\text{mg protein}$.

2.4H. GDP-Binding Assay.

Specific binding of GDP to brown adipose tissue mitochondria was measured by the method of Rial and Nicholls (1983).

Figure 2.3. Figure 2.3a shows GDP-binding to mitochondria from normal, warm-acclimated rats (see GDP-binding section for details of procedure). $n = 3$ for each point. From the curve the maximal binding conditions were established and used to investigate the effect of 3 days BRL 26830A treatment on maximal GDP-binding to BAT mitochondria (see results figure 3.1). Figure 2.3b shows a Scatchard analysis of the GDP-binding curve in figure 2.3a.

Figure 2.3a. GDP-binding to BAT mitochondria from Warm-acclimated Rats.

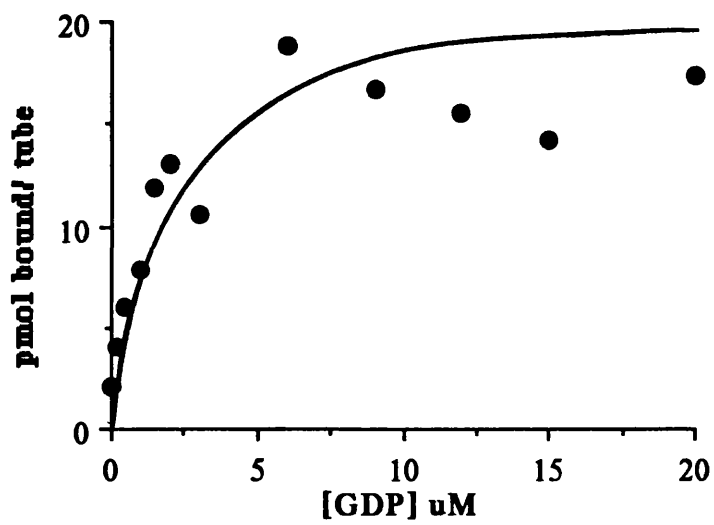
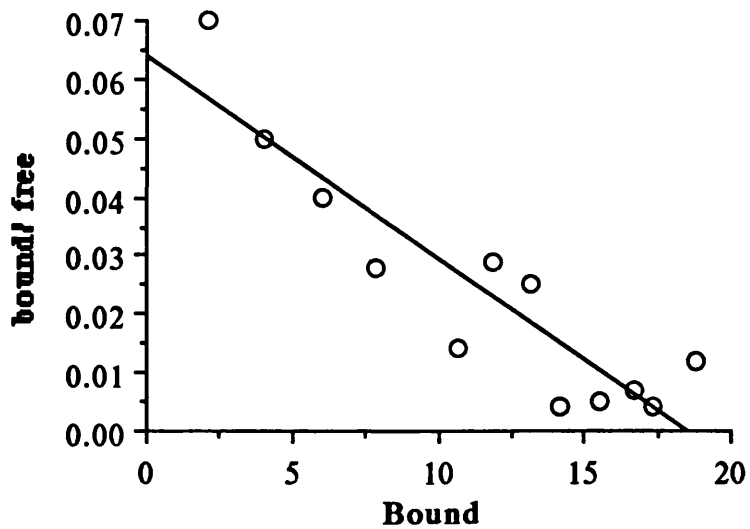


Figure 2.3b. Scatchard Analysis of GDP-binding to BAT mitochondria.



Mitochondria (prepared as described above) (0.3 mg/ml) were incubated for 5 min. at 30°C in a final volume of 250 μ l of 100 mM KCl, 5 mM Tris-HCl, pH 7.0, 2.5 μ M rotenone, 0.1 μ Ci [14 C]sucrose and 0.75 μ Ci [3 H]GDP. Varying concentrations of unlabelled GDP were added (0-25 μ M) and non-specific binding was measured in the presence of 1 mM GDP. After incubation the reaction was terminated by centrifugation at 800g in a bench microfuge. The supernatant was discarded and the tubes dried (inverted on tissue paper for 30 min.). The pellets were then solubilised with 10% Triton X-100 (0.5 ml, 30 min. at 37°C) and the solution added to 10 ml of Ecoscint A[®] for dual-label scintillation counting (Packard CR 1600) of the radioactivity associated with the mitochondria.

Association of GDP with the mitochondria was calculated after allowing for the extra-matrix volume trapped in the assay as estimated by the [14 C]sucrose space. Correction for non-specific binding was applied to all samples. Specific GDP-binding was expressed as pmol bound/mg protein

GDP-binding in the presence and absence of BRL-26830A injections in test animals, was measured in the presence of 25 μ M unlabelled GDP as this was found to give maximal binding (from the above binding curve, see figure 3). Specific GDP-binding was expressed as pmol bound/mg protein.

2.5. Scintillation Counting.

The radioactively labelled products produced in the above assays was measured using a Packard CR 1600 scintillation counter which had been preprogrammed for counting 14 C and 3 H isotopes, either singly or together. The internal computer had quench curves stored, enabling direct printing of disintegrations per minute (DPM).

2.6. Statistical Analysis.

The n value expressed for each group signifies the number of animals in that group, or the number of preparations (*e.g.* GDP-binding). The statistical significance between different groups was measured using Student's t test for paired or unpaired samples.

RESULTS AND
DISCUSSION.
CHAPTER THREE.

3.1. Effect of Adrenergic Agonists and Cold-Acclimation on the Activities of the Glycerolipid Synthesis Enzymes in Brown Adipose Tissue.

Darnley *et. al.* (1988) reported that exposure to cold for 1-12 days significantly increased the activities of both mitochondrial and microsomal GPAT, Mg²⁺-dependant PPH, MGPAT and FAS. Acute stimulation of BAT results in the mobilization of intracellular stores of triacylglycerol to provide the fuel for thermogenesis. After 24 h. these stores are exhausted and fatty-acids are supplied by lipogenesis and the action of LPL on circulating VLDL and chylomicrons. At about 3 days of stimulation tissue "recruitment" begins, with increases in mitochondriogenesis and cellular proliferation. Also at this time triacylglycerol stores begin to be replenished. Both of the above require glycerolipid synthesis, (phospholipid production is an essential part of membrane synthesis required for mitochondrial and cellular proliferation) thus changes in the activities of the enzymes of the glycerolipid synthesis pathway in BAT could be seen as an early event in tissue recruitment. With this in mind it was decided to further investigate the effect of cold stimulation of BAT on the glycerolipid synthesis enzymes at the ^{transitional} period of 3 days stimulation. The signals driving cold-stimulation of BAT are generally thought to be mediated by noradrenaline released from the sympathetic nervous system, although many other hormonal effects have been reported. The effect of adrenergic stimulation on the activities of the glycerolipid synthesis enzymes has not been studied previously, hence the studies presented here. Both α and β -adrenergic-mediated pathways are involved in BAT stimulation. In the following studies phenylephrine was used as an α -adrenergic agonist and BRL-26830A as a β_3 -specific β -adrenergic agonist. BRL-26830A has been reported to stimulate BAT thermogenesis and lipolysis (Arch *et. al.*, 1984; Wilson, 1989). To test whether BRL-26830A was able to mimic the effect of cold at the dose used (1 mg/kg body weight), the effect of 3 days BRL-26830A treatment on maximal GDP-binding to BAT mitochondria from warm-adapted normal rats was studied. The treatment resulted in a significant increase in maximal GDP-binding to BAT mitochondria (Figure 3.1) which

Figure 3.1. Effect of 3 days Treatment with BRL 26830A on maximal GDP-binding to Brown Adipose Tissue mitochondria from warm-acclimated rats.

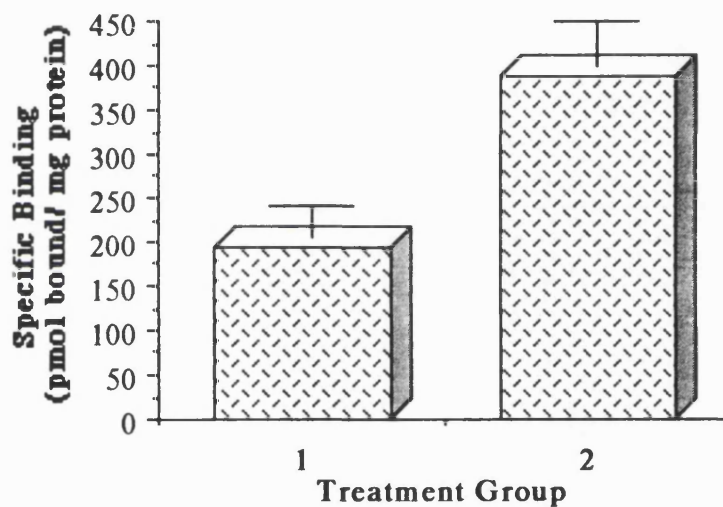


Figure 3.1. Figure 3.1 illustrates the effect of 3 days treatment with BRL 26830A on maximal GDP-binding to mitochondria from warm-acclimated, normal rats. The maximal binding conditions used are listed in the Materials and Methods section. Specific binding is given as pmol bound/ mg protein and $n = 4$ for each group. $p < 0.01$ for control (group 1) versus BRL-treated (group 2), indicating a significant increase in GDP-binding in the BRL-treated group.

indicates that BRL-26830A was able to elevate the thermogenic capacity of BAT in a similar manner to cold-exposure. Thus it was possible to use the stated dose of BRL-26830A to investigate the effect of β -adrenergic stimulation of BAT and compare these effects to that of the physiological stimulus, cold.

The effect of BRL 26830A over a 3 day period on the activities of the glycerolipid synthesis enzymes was studied, with measurements taken at 6, 24, 48, and 72 h. BRL 26830A was administered at 0, 24 and 48 h. as stated in Materials and Methods. Figure 3.2 shows the effect of the treatment on mitochondrial GPAT (Figure 3.2a), PPH (figure 3.2b), FAS (Figure 3.2c), MGPAT (Figure 3.2d) and microsomal GPAT (Figure 3.2e). Some non-significant variation in enzyme activities are seen. One significant increase ($p < 0.01$) is that of MGPAT at 2-days treatment, returning to previous activity levels at 3-days (Figure 3.2d). The increase in microsomal GPAT activity from the level at 1-day to that at 3-days (Figure 3.2e) is also significant ($p < 0.01$), suggesting that stimulation of β -adrenergic-receptors may be sufficient to mediate changes in the activity of this enzyme in BAT.

The effect of BRL-26830A on BAT glycerolipid synthesis enzymes was directly compared to that of cold-exposure for 3-days. Also the effect of phenylephrine alone or in addition to BRL-26830A (the physiological effector of BAT is noradrenaline which binds to both α and β -adrenergic receptors, hence the experimental combination of BRL-26830A and phenylephrine) was investigated. The aim of the experiment was to investigate whether adrenergic stimulation of BAT could mimic the effect of cold, indicating that the adrenergic-mediated pathways are responsible for regulating glycerolipid synthesis in BAT. Table 3.1 shows the growth profiles of the rats during the experimental period and details of the interscapular BAT pads from the different treatments. Cold-exposure reduced the weight gain seen at 21°C in the untreated animals (Table 3.1a, groups 2 and 1 respectively). Administration of BRL-26830A did not mimic the effect of cold in the warm-adapted animals (Table 3.1a, group 3) but enhanced the effect of cold (Table 3.1a, group 4). Phenylephrine had no effect on weight gain when administered alone or with BRL-26830A (BRL) to warm-adapted animals (Table 3.1a, groups 5 and 6 respectively). Cold-exposure

Figure 3.2. Effect of BRL-26830A on the Activities of the Glycerolipid Synthesis Enzymes over 3-Days.

Figure 3.2 shows the effect of BRL 26830A on glycerolipid synthesis enzyme activities (expressed as nmol/ min/ 100 μ g DNA) in brown adipose tissue from warm-acclimated (21°C from birth), normal rats. Dose and injections were as described in materials and methods. Activities were measured at time 0 (animals sacrificed immediately after injection), 6 h., 1 day, 2 days and 3 days of treatment. n = 6 for each time point and error bars indicate the standard error of the mean of that point. Figure 3.2a shows variation in the activity of mitochondrial glycerolphosphate acyltransferase (GPAT). Figure 3.2b shows variation in Mg²⁺-dependant phosphatidate (PPH) activity. Figures 3.2c, 3.2d and 3.2e show variation in the activities of long-chain acyl-CoA synthase (FAS), monoacyl-glycerolphosphate acyltransferase (MGPAT) and microsomal GPAT respectively.

Figure 3.2a. Effect of BRL 26830A on mitochondrial GPAT Activity over 3 days.

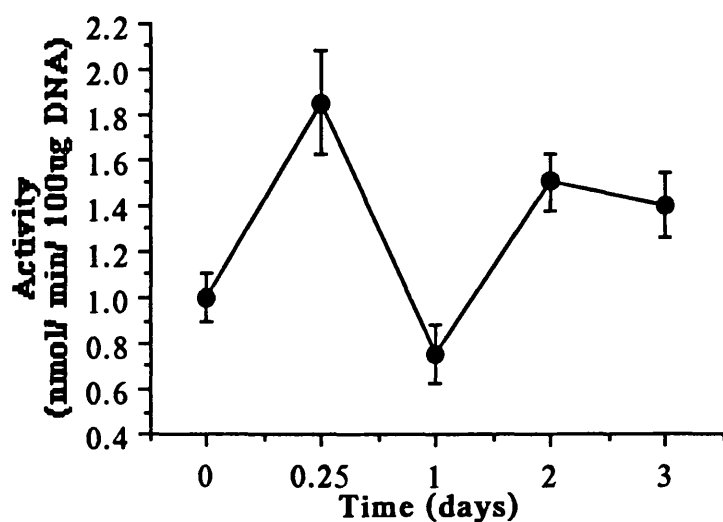


Figure 3.2b. Effect of BRL 26830A on PPH Activity over 3 days.

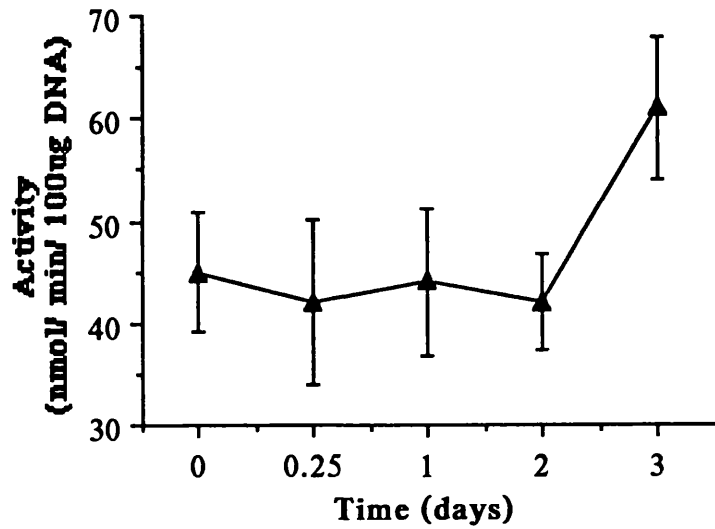


Figure 3.2c. Effect of BRL 26830A on FAS Activity over 3 days.

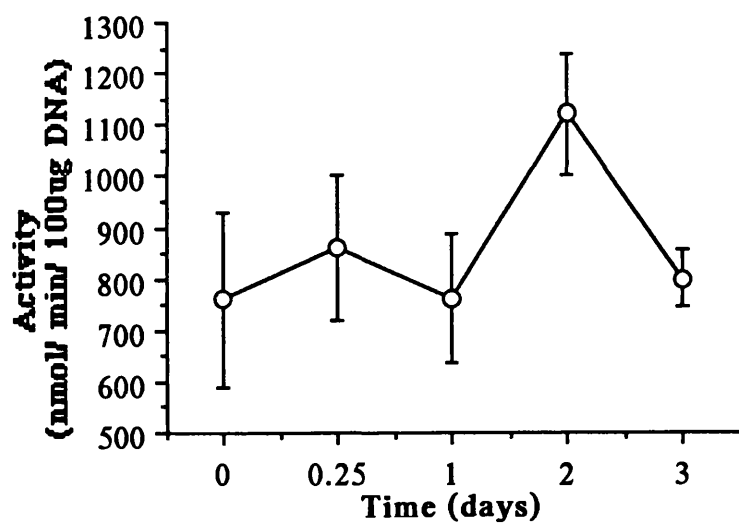


Figure 3.2d. Effect of BRL 26830A on MGPAT Activity over 3 days.

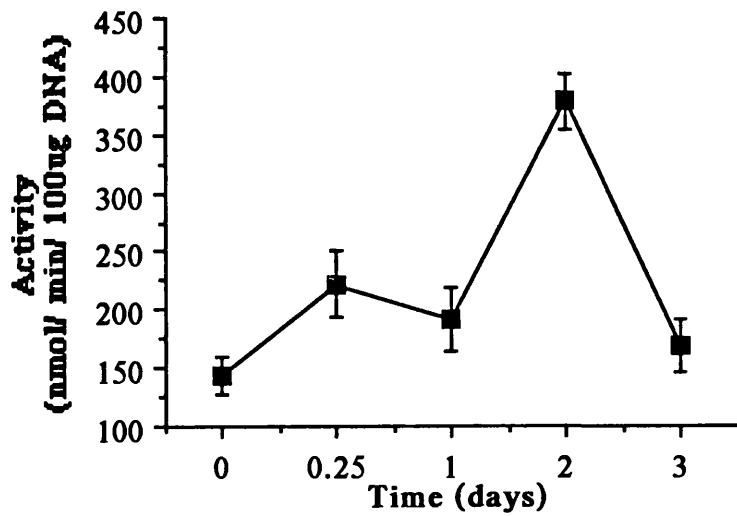
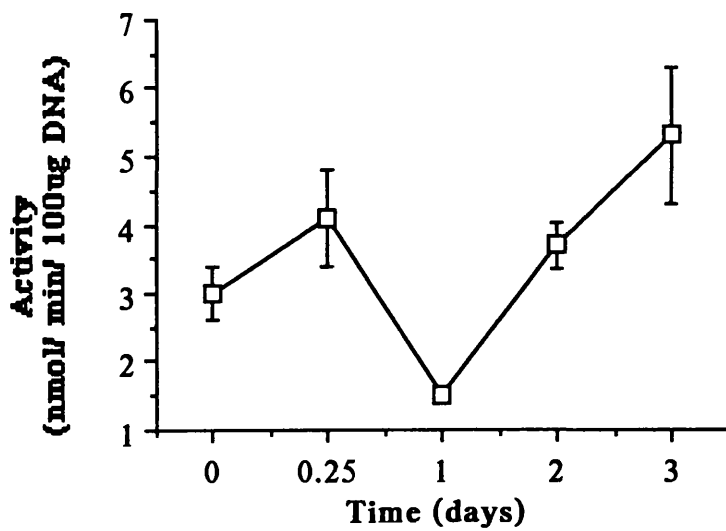


Figure 3.2e. Effect of BRL 26830A on microsomal GPAT Activity over 3 days.



resulted in increased BAT wet weight and total tissue protein, which was mimicked, but not enhanced, by administration of BRL (Table 3.1b). The combination of phenylephrine and BRL did not enhance the effect of BRL alone (Table 3.1b). The increase in wet weight and total protein indicates that tissue hypertrophy has been initiated and it appears that only β -adrenergic stimulation is required to initiate this aspect of BAT recruitment. Total tissue DNA was not significantly increased by any of the above treatments. These results are similar to those reported previously (Darnley *et. al.*, 1988) except that DNA was reported to be significantly elevated at 3 days cold-exposure in the earlier report. One possible explanation of this discrepancy is that in the earlier report animals were housed 4 per cage and in this study they were housed 6 per cage which might reduce the degree of "cold" experienced and thus reduce any visible effects slightly. Also the time point at which these measurements was made occurs at the threshold of measurable cell proliferation which, therefore, may or may not be reflected in significant increases in tissue DNA.

Table 3.2a, b and Figure 3.3a-e show the effects of cold, BRL at 21°C and 4°C, phenylephrine and BRL + phenylephrine on the activities (Table 3.2a, nmol/min/100 μ g DNA; Table 3.2b, nmol/min/mg protein) of the glycerolipid synthesis enzymes. The Tables shown here (and in section 3.2) list enzyme activities as both nmol/min/100 μ g DNA and nmol/min/mg protein. Protein amount per cell within a tissue will vary, especially in a tissue at the threshold of mitochondriogenesis and cellular proliferation as is the case in these studies. However cellular DNA content is invariable and comparisons made in relation to the level of DNA therefore more accurate. Thus in discussing changes in activity of the enzymes studied, reference is only made to activity values expressed as nmol/min/100 μ g DNA. This applies to all the studies on the glycerolipid synthesis enzymes in BAT presented below.

Cold-exposure significantly elevated the activities of mitochondrial GPAT, PPH, FAS and microsomal GPAT. The increase in mitochondrial GPAT was particularly pronounced, an approximate 5-fold increase over the warm-adapted level.

Administration of BRL failed to mimic the effect of cold-exposure except in the case

Tables 3.1-3.10. For tables 3.1-3.10, all treatments were as stated in Materials and Methods section. "None" indicates the appropriate control to the following treatment in the relevant table. Statistical analysis is shown and explained on the tables, n values are also listed.

Figures 3.3-3.7. Figures 3.3-3.7 relate directly to tables 3.2a, 3.4a, 3.6, 3.8 and 3.10 and show the percentage changes in enzyme activities (nmol/ min/ 100 μ g DNA only) from those tables. The group number shown relates directly to the group number used on the corresponding table. Control group activities are arbitrarily given the value of 100% and the other activities related to that value. In figures 3.4 and 3.7 two groups are given the 100% value as they are control groups within the corresponding tables, *e. g.* in figure 3.7 groups 2-4 are compared to group 1 and groups 6-8 are compared to group 5. Figure 3.4 also has groups 1 and 5 as 100% values, with group 6 (hyperthyroid) compared to its appropriate control group (group 5) and the other groups compared to group 1.

As these figures are "normalised" they are presented as an illustration of the changes in enzyme activities and are not quantitative. Each figure represents one enzyme; 3.3a, 3.3b, 3.3c, 3.3d and 3.3e show changes in mitochondrial GPAT, PPH, MGPAT, FAS and microsomal GPAT respectively and this labelling applies to all the figures (up to and including figure 3.7).

Table 3.1. Effect of Cold-Acclimation and Adrenergic Agonists on Rat Weights and Interscapular Brown Adipose Tissue from Normal Rats.

Table 3.1a. Body weights of rats during treatments and cold-exposure.

Treatment	Temp (°C)	n	Body wt. during treatment and/or cold-exposure	
			at start	at end
1 None	21	13	162 ± 5	180 ± 5 ^A
2 None	4	6	183 ± 2	187 ± 1
3 BRL 26830A	21	12	166 ± 6	187 ± 3 ^B
4 BRL 26830A	4	6	187 ± 2	179 ± 1 ^A
5 Phenylephrine	21	6	177 ± 11	202 ± 14
6 Phenylephrine+ BRL 26830A	21	6	174 ± 9	188 ± 12

^{A,B}Indicates $P < 0.05$, 0.02 respectively for the change in animal weight over the experimental period.

Table 3.1b. Details of interscapular adipose tissues from all groups studied.

For comparison of conditions 2-6 versus 1, ^{A, B, C}indicates $P < 0.02$, 0.01 , 0.001 respectively. ^Dindicates $P < 0.05$ for comparison of 4 with 3. ^Eindicates $P < 0.02$ for comparison of 6 with 5.

Treatment	Temp (°C)	n	Interscapular brown-fat content of:		
			Wet wt. (g)	Protein (mg)	DNA (µg)
1 None	21	13	0.34 ± 0.01	13.1 ± 0.5	178 ± 17
2 None	4	6	0.44 ± 0.02 ^C	16.4 ± 0.8 ^B	253 ± 34
3 BRL 26830A	21	12	0.50 ± 0.02 ^C	17.3 ± 0.9 ^C	188 ± 12
4 BRL 26830A	4	6	0.43 ± 0.02 ^{C, D}	16.2 ± 1.0 ^A	261 ± 37
5 Phenylephrine	21	6	0.42 ± 0.04	12.8 ± 0.6	209 ± 31
6 Phenylephrine+ BRL 26830A	21	6	0.50 ± 0.03 ^C	15.4 ± 0.6 ^{B, E}	259 ± 46

Table 3.2a. Effects of cold-acclimation and adrenergic agonists on enzyme activities in brown adipose tissue from normal rats.

For 2-6 vs 1, A, B, C, D indicate $P < 0.05$, 0.02 , 0.01 , 0.001 respectively. E, G, H indicate $P < 0.05$, 0.01 , 0.001 respectively for comparison of 4 with 3. There was no significant difference ($P > 0.05$) in every case between 5 and 6. For group 3, * indicates $n = 10$ and $^{\circ}$ indicates $n = 11$. For groups 4, + indicates $n = 5$.

Treatment	Temp (°C)	n	Enzyme activity (nmol /min per 100 μ g of tissue DNA).					
			mitochondrial GPAT	PPH	microsomal GPAT	FAS	MGPAT	NADP-cytochrome c-reductase
1 None	21	13	2.0 \pm 0.3	108 \pm 12	2.9 \pm 0.4	883 \pm 79	121 \pm 13	50.2 \pm 8.7
2 None	4	6	10.6 \pm 1.5 ^D	195 \pm 30 ^B	5.8 \pm 0.6 ^D	1669 \pm 231 ^C	152 \pm 40	52.2 \pm 4.9
3 BRL 26830A	21	12	2.8 \pm 0.3 [*]	81 \pm 8	5.3 \pm 1.0 ^A	800 \pm 54	171 \pm 23 ^o	55.2 \pm 4.5
4 BRL 26830A	4	6	8.2 \pm 1.2 ^{D, H}	205 \pm 38 ^{A, G}	6.8 \pm 1.2 ^C	1913 \pm 292 ^{C, G}	294 \pm 48 ^{C, E +}	67.5 \pm 10.7
5 Phenylephrine	21	6	0.4 \pm 0.1 ^D	69 \pm 8 ^B	1.4 \pm 0.2 ^C	586 \pm 122 ⁺	73 \pm 15 ^A	29.0 \pm 7.2
6 Phenylephrine+ BRL 26830A	21	6	0.6 \pm 0.2 ^C	66 \pm 12 ^A	1.4 \pm 0.3 ^C	909 \pm 231	74 \pm 18 ^A	30.3 \pm 3.6 ^A

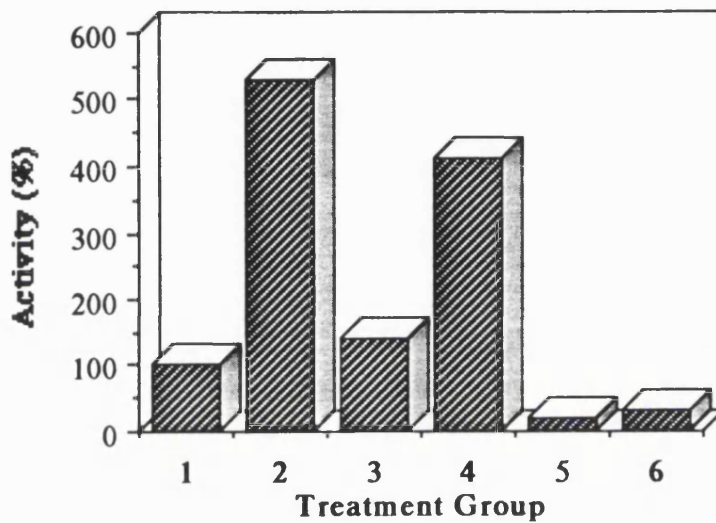
Table 3.2b. Effects of cold-acclimation and adrenergic agonists on enzyme activities in brown adipose tissue from normal rats.

For 2-6 vs 1, A, B, C, D indicate $P < 0.05, 0.02, 0.01, 0.001$ respectively. E, F, G, H indicates $P < 0.05, 0.02, 0.01, 0.001$ respectively for comparison of 4 with 3. There was no significant difference ($P > 0.05$) in every case between 5 and 6. For group 3, * indicates $n = 10$ and ^o indicates $n = 11$. For groups 4, + indicates $n = 5$.

Treatment	Temp (°C)	n	Enzyme activity (nmol /min per mg protein).					
			mitochondrial GPAT	PPH	microsomal GPAT	FAS	MGPAT	NADP-cytochrome c-reductase
1 None	21	13	0.24 ± 0.02	13.4 ± 0.9	0.37 ± 0.04	110 ± 6.0	15.5 ± 1.5	5.8 ± 0.8
2 None	4	6	1.45 ± 0.06 ^D	26.5 ± 1.1 ^D	0.82 ± 0.06 ^D	232 ± 13 ^D	20.0 ± 4.0	7.0 ± 0.4
3 BRL 26830A	21	12	0.29 ± 0.03 ⁺	8.3 ± 0.41 ^D	0.51 ± 0.1 ^B	87 ± 7.6 ^A	17.2 ± 1.6 ^o	6.2 ± 0.8
4 BRL 26830A	4	6	1.21 ± 0.12 ^{DH}	28.6 ± 1.0 ^{DH}	1.06 ± 0.22 ^{DF}	279 ± 28 ^{DH}	40.0 ± 3.0 ^{+DH}	9.7 ± 1.0
5 Phenylephrine	21	6	0.05 ± 0.01 ^D	10.3 ± 0.6 ^B	0.18 ± 0.01 ^D	104 ± 14 ⁺	10.2 ± 1.2 ^B	4.1 ± 0.5
6 Phenylephrine+ BRL 26830A	21	6	0.09 ± 0.03 ^D	9.1 ± 0.1 ^D	0.2 ± 0.03 ^D	116 ± 16	10.2 ± 0.9 ^B	5.9 ± 0.8

**Figure 3.3. Relative Activities of the Glycerolipid Synthesis
Enzymes using Activity Values from Table 3.2a.**

**Figure 3.3a. Relative Activity of mitochondrial GPAT using Activity values
from Table 3.2a.**



**Figure 3.3b. Relative Activity of PPH using Activity values from Table
3.2a.**

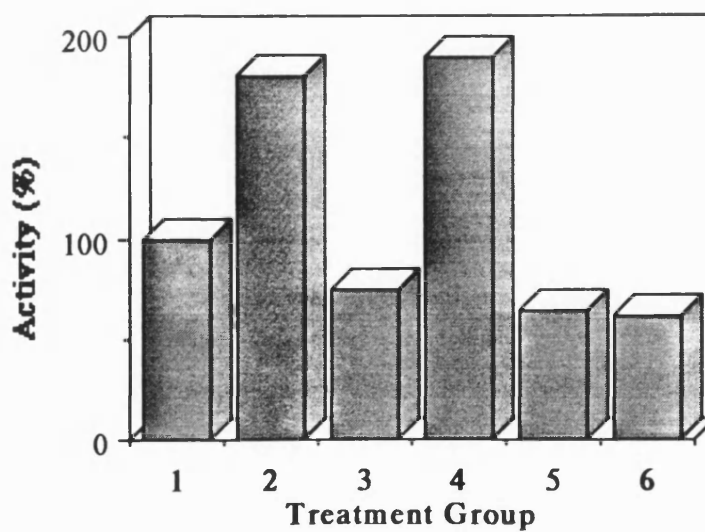


Figure 3.3c. Relative Activity of FAS using Activity values from Table 3.2a.

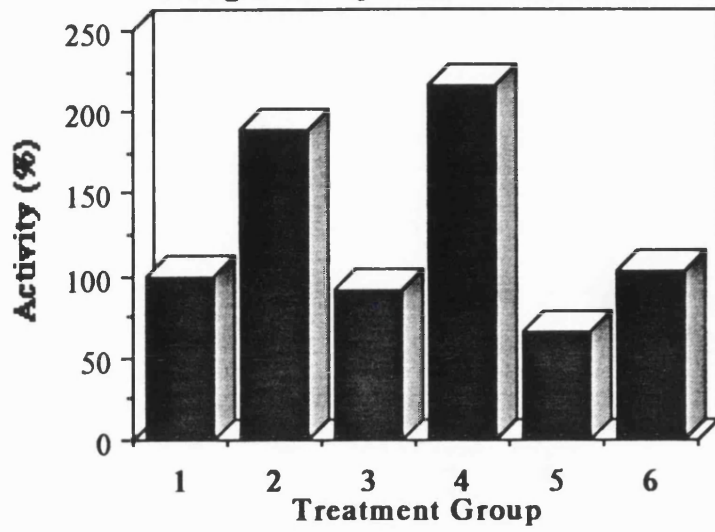


Figure 3.3d. Relative Activity of MGPAT using Activity values from Table 3.2a.

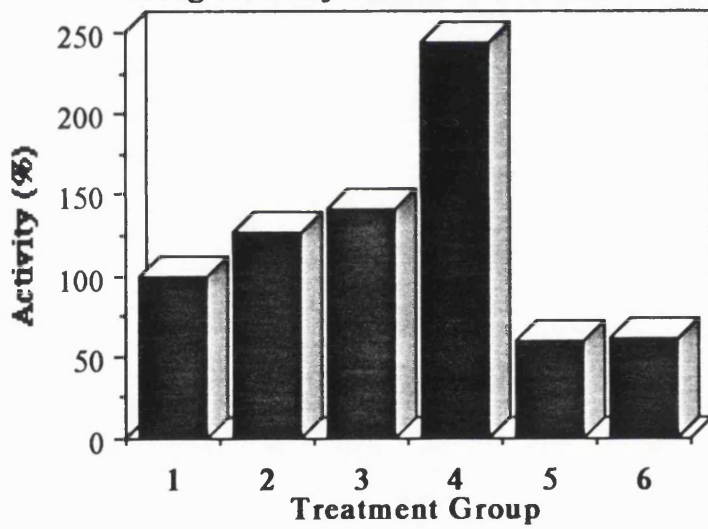
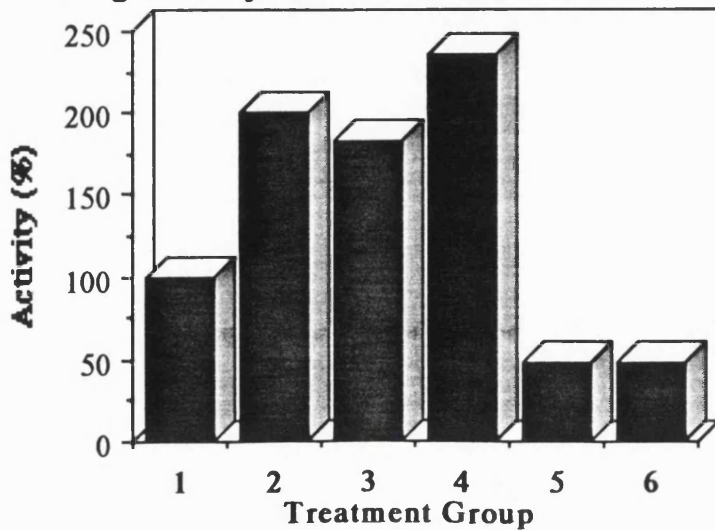


Figure 3.3e. Relative Activity of microsomal GPAT using Activity values from Table 3.2a.



of microsomal GPAT. BRL also did not enhance the cold-induced increase in enzyme activity, except in the case of MGPAT where only BRL administration and cold-exposure significantly elevated enzyme activity.

Phenylephrine administration significantly decreased the activities of mitochondrial GPAT, microsomal GPAT, PPH and MGPAT. The decrease was between 40 and 80% (PPH and mitochondrial GPAT respectively) and was not overcome by addition of BRL.

The activity of NADP⁺-cytochrome *c*-reductase is listed as a marker for the microsomal fraction of the cell. The microsomal fraction of BAT contains all the glycerolipid synthesis enzymes except mitochondrial GPAT and the predominant fraction of FAS (Baht and Saggerson, 1988a), so any change in the relative amount of the microsomal fraction may reflect changes in the enzyme activities per mg protein associated with this fraction. However no significant change in NADP⁺-cytochrome *c*-reductase was recorded, although phenylephrine reduced the activity in a non-significant manner. This indicates the increase in activities recorded are over and above any increase in the level of protein associated with the microsomal fraction during any increase in total tissue mass.

The above results are similar to those reported by Darnley *et. al.*, (1988) in terms of the effect of cold-exposure. It appears that adrenergic stimulation of BAT is not sufficient to mimic the effect of cold-exposure on the enzymes studied, except in the case of microsomal GPAT. This observation parallels with that of Baht and Saggerson (1988a) who reported no acute effect of noradrenaline on the activity of these enzymes in brown adipocytes. However cold-induced increase in microsomal GPAT appears to be mediated by the β -adrenergic pathways in BAT, suggesting regulatory differences between the two forms of GPAT in this tissue. The α -adrenergic pathways are definitely not involved in the cold-induced increases in enzyme activities and are highly inhibitory. Whether the failure of BRL to overcome the effects of phenylephrine, especially on the activity of microsomal GPAT, is physiological or due to a large overdose of the α -adrenergic agonist is not clear. In white adipose tissue (WAT) adrenergic stimulation causes reduction in the activities of

FAS, PPH and GPAT (Sooranna and Saggerson, 1978) which would correspond to the above effects of BRL+phenylephrine.

The physiological signal for stimulation of BAT is considered to be mediated via noradrenaline acting on the adrenergic receptors but there are numerous hormonal aspects also involved (see Introduction for details). Thus the effect of altered hormonal status on cold-induction of the glycerolipid synthesis enzymes was investigated with the aim of eliciting whether any of the hormones affecting thermogenesis in BAT also regulated this aspect of BAT metabolism.

3.2. Effect of Hormonal Status on the Cold-induced Changes in Glycerolipid Synthesis Enzyme Activities in Brown Adipose Tissue.

3.2A. Effect of Adrenergic Stimulation and Cold-acclimation on the Activities of the Glycerolipid Synthesis Enzymes from Diabetic Rats.

Diabetes has been reported to result in lesions in thermogenesis in BAT at the level of thermogenin production/activity (Jamal and Saggerson, 1988; Seydoux *et. al.*, 1984) and fatty acid oxidation (Gualberto and Saggerson, 1989; Jamal and Saggerson, 1988b; Seydoux *et. al.*, 1983). Insulin appears therefore to have a role in regulation of BAT metabolism, which prompted the investigation of the effect of insulin status on the cold-induced increases in the activities of the glycerolipid synthesis enzymes in this tissue. It has been demonstrated that the effects of diabetes on BAT mitochondria can be overcome by insulin treatment (Gualberto and Saggerson, 1989) but that some aspects of insulin action are secondary to the adrenergic regulation of the tissue (Jamal and Saggerson, 1988). Therefore the effect of the β_3 -adrenergic agonist BRL-26830A (BRL) on glycerolipid synthesis enzyme activities in diabetic rats was investigated.

Diabetes was induced prior to cold-exposure or treatment with BRL as stated in Materials and Methods. The diabetic state of the animals was confirmed by measurement of blood glucose levels and testing for glucose in the urine. Diabetic animals all gave positive

Table 3.3. Effect of Cold-Acclimation and BRL-26830A on Rat Weights and Interscapular Brown Adipose Tissue from Diabetic Rats.

Table 3.3a. Body weights of rats during treatments and cold-exposure.

Treatment	Temp (°C)	n	Body wt. during treatment and/or cold-exposure	
			at start	at end
1 None	21	6	244 ± 11	258 ± 12
2 Diabetic	21	6	217 ± 9	218 ± 10
3 Diabetic	4	6	214 ± 5	207 ± 5
4 Diabetic+	21	6	203 ± 9	209 ± 13

BRL 26830A

Table 3.3b. Details of interscapular adipose tissues from all groups studied.

For comparison of 2 versus 1, ^Aindicates $P < 0.05$. For comparison of 3 and 4 versus 2, ^B, ^Cindicates $P < 0.02$, 0.001 respectively.

Treatment	Temp (°C)	n	Interscapular brown-fat content of:		
			Wet wt. (g)	Protein (mg)	DNA(mg)
1 None	21	6	0.27 ± 0.03	15.2 ± 0.9	415 ± 57
2 Diabetic	21	6	0.24 ± 0.02	12.2 ± 0.8 ^A	289 ± 24
3 Diabetic	4	6	0.20 ± 0.01	10.3 ± 1.0	504 ± 76 ^B
4 Diabetic+	21	6	0.22 ± 0.02	11.0 ± 1.2	518 ± 67 ^C

BRL 26830A

Table 3.4a. Effects of cold-acclimation and BRL 26830A on enzyme activities in brown adipose tissue from diabetics.

For comparison of 2 versus 1, ^Aindicates $P < 0.05$. For comparison of 3 and 4 versus 2, ^B, ^C, ^Dindicates $P < 0.02$, 0.01 , 0.001 respectively.

Treatment/ Animal Status	Temp (°C)	n	Enzyme activity (nmol /min per 100 µg of tissue DNA).				
			mitochondrial GPAT	PPH	microsomal GPAT	FAS	MGPAT
1 None/ normal	21	6	0.9 ± 0.2	38 ± 8	1.0 ± 0.2	596 ± 62	163 ± 28
2 None/ Diabetic	21	6	0.3 ± 0.1 ^A	31 ± 9	0.6 ± 0.1	636 ± 114	168 ± 24
3 None/ Diabetic	4	6	2.3 ± 0.4 ^D	20 ± 4	2.0 ± 0.2 ^D	479 ± 44	335 ± 46 ^C
4 BRL 26830A/ Diabetic	21	6	0.3 ± 0.1	19 ± 7	1.3 ± 0.2 ^B	574 ± 79	144 ± 11

Table 3.4b. Effects of cold-acclimation and BRL 26830A on enzyme activities in brown adipose tissue from diabetics.

For comparison of 2 versus 1, ^Aindicates $P < 0.05$. For comparison of 3 and 4 versus 2, ^{B, C, D}indicates $P < 0.02, 0.01, 0.001$ respectively. For group IV, ⁺indicates $n = 5$.

Treatment/ Animal Status	Temp (°C)	n	Enzyme activity (nmol /min per mg protein).				
			mitochondrial GPAT	PPH	microsomal GPAT	FAS	MGPAT
1 None/ Normal	21	6	0.22 ± 0.03	7.3 ± 1.1	0.25 ± 0.04	156 ± 14	41.0 ± 3.9
2 None/ Diabetic	21	6	0.06 ± 0.02 ^A	6.6 ± 1.5	0.14 ± 0.03	143 ± 18	40.7 ± 6.9
3 None/ Diabetic	4	6	1.03 ± 0.12 ^D	8.6 ± 0.7	0.93 ± 0.11 ^D	225 ± 28 ^B	154 ± 21.0 ^D
4 BRL 26830A/ Diabetic	21	6	0.14 ± 0.02 ^B	8.3 ± 2.4	0.60 ± 0.08 ^D	264 ± 25 ^C	71.4 ± 7.1 ^{B+}

Figure 3.4. Relative Activities of the Glycerolipid Synthesis Enzymes using Activity Values from Table 3.4a.

Figure 3.4a. Relative Activity of mitochondrial GPAT using Activity values from Table 3.4a.

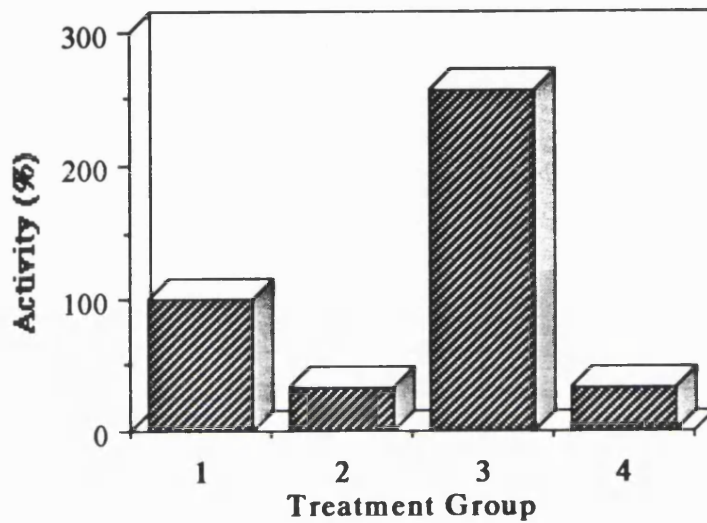


Figure 3.4b. Relative Activity of PPH using Activity values from Table 3.4a.

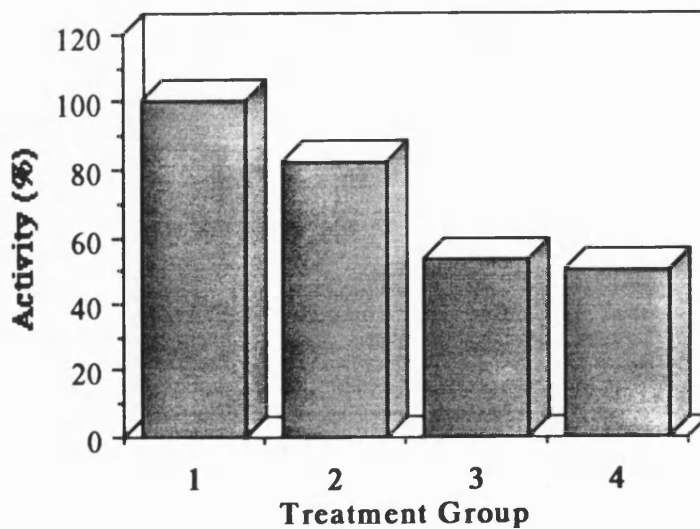


Figure 3.4c. Relative Activity of FAS using Activity values from Table 3.4a.

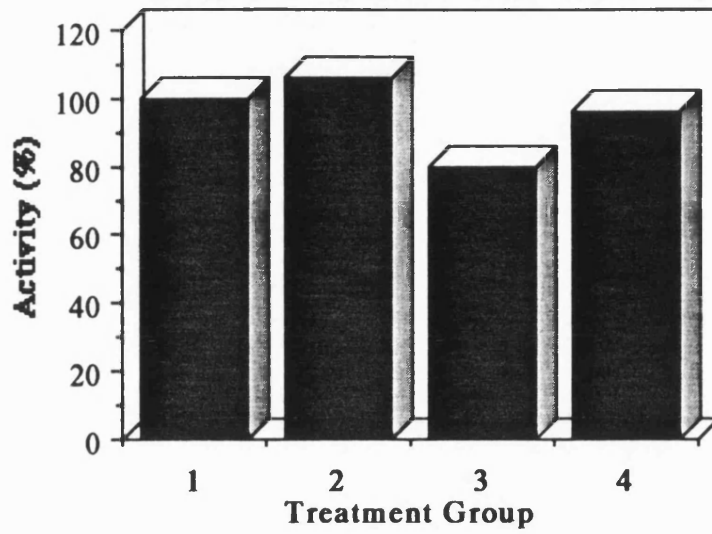


Figure 3.4d. Relative Activity of MGPAT using Activity values from Table 3.4a.

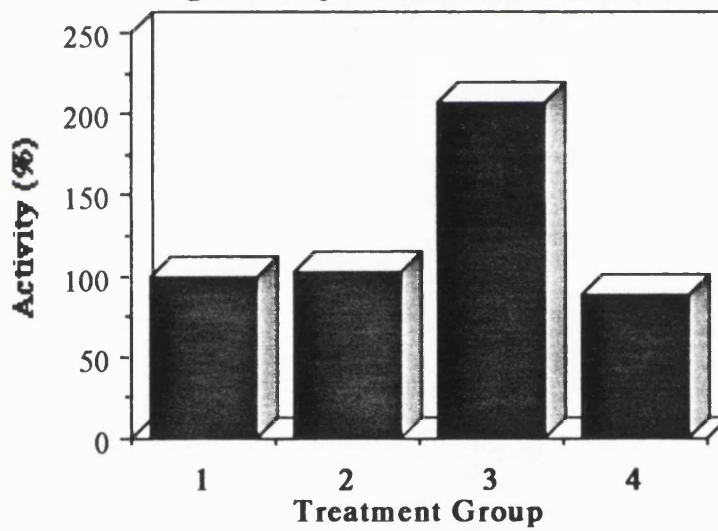
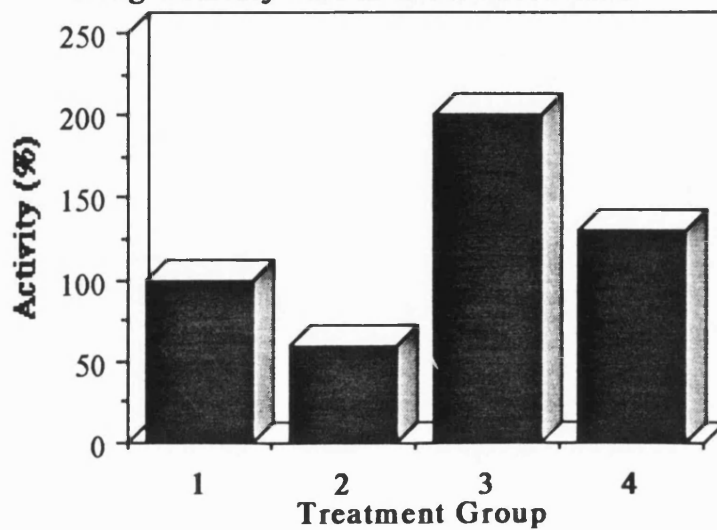


Figure 3.4e. Relative Activity of microsomal GPAT using Activity values from Table 3.4a.



results in the Clinistix® test of glucose in the urine and displayed significantly elevated blood glucose as compared to normal animals (27.1 ± 1.1 mM blood glucose versus 7.7 ± 0.25 mM in diabetics and normals respectively).

Diabetes prevented the weight-gain seen in normal rats at 21°C over 3-days. Cold-exposed diabetic rats lost weight (Group 3, Table 3.3a) in a similar manner to that seen in normal rats (Group 3, Table 3.1a; Darnley *et. al.*, 1988). Diabetics treated with BRL showed approximately 50% of the normal weight-gain at 21°C (Group 4, Table 3.3a). Diabetes also caused a reduction in total tissue protein and total tissue DNA (approximately 30%) (Group 2, Table 3.3b) which indicates atrophy of BAT. This has been reported previously for BAT during chronic diabetes (Bartness *et. al.*, 1986a, b; Jamal and Saggerson, 1988; Seydoux *et. al.*, 1983). Interestingly cold-exposure significantly increased total tissue DNA in the diabetic animals, indicating that the capacity for BAT recruitment had not been impaired by this short-term (6 days) diabetes (Group 3, Table 3.3b). Administration of BRL was able to mimic the cold-induced increase in total tissue DNA in warm-adapted diabetic rats (Group 4, Table 3.3b), indicating that this aspect of BAT tissue recruitment is under adrenergic regulation only. This observation complements the report of Geleon *et. al.*, (1988) who stated that chronic β -adrenergic stimulation of BAT was sufficient to mimic cold-induced tissue hypertrophy.

Table 3.4a,b and Figure 3.4a-e show the effect of cold-exposure for 3 days or BRL treatment on the activity of the glycerolipid synthesis enzymes in diabetic rats. Diabetes induced a statistically significant 70% decrease in mitochondrial GPAT activity but did not significantly effect the activity of any other enzyme measured, microsomal GPAT showing a non-significant 40% decrease in activity (Group 2, Table 3.4a). Diabetes did not prevent the cold-induced increase in mitochondrial GPAT activity (8-fold increase) or microsomal activity (3-fold increase), but abolished the cold-induced increases in PPH and FAS activity (Group 3, Table 3.4a, and Figure 3.4a, 3.4e, 3.4b and 3.4c respectively) seen previously in normal rats (Table 3.2a). BRL treatment did not effect enzyme activities except in the

case of microsomal GPAT where the administration of the β_3 -adrenergic agonist mimicked the effect of cold (Group 4, Table 3.4a), which is also seen in normal rats (Table 3.2a). In contrast to normal rats, cold-exposure in diabetic rats significantly increased the activity of MGPAT (Group 3, Table 3.4a).

The above results indicate that, although there appears to be a requirement for insulin to maintain basal levels of GPAT, insulin is not the signal mediating the cold-induced increases in activity of the two forms of GPAT. This is in contrast to the effect of insulin on white adipose tissue GPAT which has been reported to be elevated by insulin (Vila and Farese, 1991). The effect of BRL on microsomal GPAT further indicates that the activity of this form of GPAT is regulated via the β -adrenergic pathways in BAT. The results also suggest that the cold-induced increases in PPH and FAS are regulated via an insulin-mediated mechanism as diabetes prevented the increase in these activities in the cold. The regulation of FAS and PPH in white adipose tissue also appears to be mediated by the action of insulin (Saggerson and Carpenter, 1987; Taylor and Saggerson, 1986) but diabetes elevated liver PPH activity (Woods *et. al.*, 1981). This may indicate that PPH and FAS are regulated in a similar manner to that in WAT in BAT but GPAT appears to have a different regulatory signal to that seen in either WAT or liver.

In contrast to the above, Baht and Saggerson (1988) reported no decrease in GPAT activity and an increase in PPH activity in brown adipocytes from diabetic rats. However the method used to recover adipocytes after collagenase treatment of tissue pieces relies on floatation due to triacylglycerol content. As this is low in brown adipocytes a proper representation of the tissue may not be obtained by this method and lead to discrepancies such as the one mentioned here.

Chronic diabetes leads to a reduction in the oxidation of fatty acids (decreased thermogenesis and tissue atrophy) and the provision of those fatty acids. It appears from the above that the capacity for glycerolipid synthesis in warm-adapted animals may also be reduced, but the capacity to increase glycerolipid production, at least to the level of phosphatidate and anionic phospholipids is not impaired.

3.2B. Effect of Thyroid Status on Cold-Acclimation Induced Changes in the Activities of Two of the Glycerolipid Synthesis Pathway Enzymes in Brown Adipose Tissue.

Hypothyroidism (lack of thyroid hormones T₃ and T₄) results in impaired BAT thermogenesis similar to that seen in diabetes (Jamal and Saggerson, 1988a,b; Woodward and Saggerson, 1989). However hypothyroidism, unlike diabetes, has been reported to double the esterification potential of BAT while reducing β -oxidation of fatty acids (Baht and Saggerson, 1988; Blenneman *et. al.*, 1990; Woodward and Saggerson, 1989).

Hyperthyroidism (excess thyroid hormones) is reported to result in increased thermogenic capacity in BAT (Woodward and Saggerson, 1989), but only if the sympathetic nervous system is intact.

In the following study hypothyroidism was induced over 1 month prior to the start of further treatment. The hypothyroid group was age-matched to euthyroid controls (approximately 4 weeks of age prior to induction of hypothyroidism) and the difference in weight of the two conditions at the start of the study is a direct result of hypothyroidism. This is due to the fact that the hypothyroid animals had essentially ceased to grow at this point, which has been reported previously (Chohan *et. al.*, 1984). Animals were cold-exposed for 2 days only in this study, as longer cold-stress was not tolerated by the hypothyroid animals. Weight gain of the hypothyroid group (group 3, Table 3.5a) at 21°C was reduced by 60% as compared to the euthyroid controls. Both euthyroid and hypothyroid animals lost weight over the 2 days cold-exposure (groups 3 and 4, Table 3.5a). Hyperthyroidism caused a 90% reduction in weight-gain over 4 days at 21°C (groups 5 and 6, Table 3.5a).

Hypothyroidism resulted in significant increases in BAT wet weight and total tissue protein but not total tissue DNA. Total protein levels were significantly raised by cold-exposure in a similar manner to that seen in the euthyroid animals (Table 3.5b). Hyperthyroidism had no effect on BAT wet weight, total tissue protein or total tissue DNA

Table 3.5. Effect of Cold-Acclimation on Rat Weights and Interscapular Brown Adipose Tissue from Hypothyroid and Hyperthyroid Rats.

Table 3.5a. Body weights of rats during treatments and cold-exposure.

Treatment	Temp (°C)	n	Body wt. during treatment and/or cold-exposure	
			at start	at end
1 Euthyroid	21	4	282 ± 19	298 ± 21
2 Euthyroid	4*	4	304 ± 16	289 ± 17
3 Hypothyroid	21	5	200 ± 12	206 ± 11
4 Hypothyroid	4*	5	209 ± 12	196 ± 11
* indicates 2-days cold exposure only.				
5 Euthyroid	21	6	219 ± 19	240 ± 15
6 Hyperthyroid	21	6	208 ± 14	210 ± 16

Table 3.5b. Details of interscapular adipose tissues from all groups studied.

For comparison of 2-3 versus 1, A, B indicates $P < 0.05$, 0.01 respectively. 4 versus 2, C, D indicates $P < 0.05$, 0.01 respectively. 4 versus 3, E indicates $P < 0.05$.

Treatment	Temp (°C)	n	Interscapular brown-fat content of:		
			Wet wt. (g)	Protein (mg)	DNA(µg)
1 Euthyroid	21	4	0.32 ± 0.01	13.7 ± 0.5	192 ± 23
2 Euthyroid	4*	4	0.28 ± 0.02	19.6 ± 1.3 ^B	211 ± 18
3 Hypothyroid	21	5	0.50 ± 0.07 ^A	19.6 ± 1.5 ^B	220 ± 18
4 Hypothyroid	4*	5	0.43 ± 0.04 ^C	24.6 ± 1.5 ^{D, E}	233 ± 11
* indicates 2-days cold exposure only					
5 Euthyroid	21	6	0.39 ± 0.02	11.6 ± 1.5	424 ± 36
6 Hyperthyroid	21	6	0.38 ± 0.03	13.5 ± 1.7	296 ± 47

Table 3.6. Effects of cold-acclimation on mitochondrial GPAT and PPH activities in brown adipose tissue from hypothyroid or hyperthyroid rats.

For comparison of 2-3 versus 1, ^A, ^B indicates $P < 0.05$, 0.02 respectively. 4 versus 2, ^C indicates $P < 0.02$. 4 versus 3, ^D indicates $P < 0.05$. 6 versus 5, ^E indicates $P < 0.01$.

Treatment	Temp (°C)	n	Phosphatidate Phosphohydrolase activity as nmol per min:		Mitochondrial GPAT activity as nmol per min:	
			/mg of tissue protein	/100 µg of tissue DNA	/mg of tissue protein	/100 µg of tissue DNA
1 Euthyroid	21	4	12.1 ± 0.6	78.5 ± 6.6	0.23 ± 0.03	1.7 ± 0.3
2 Euthyroid	4*	4	8.6 ± 0.7 ^B	69.6 ± 1.6	0.67 ± 0.21	5.9 ± 1.6 ^A
3 Hypothyroid	21	5	11.2 ± 1.3	87.7 ± 7.1	0.66 ± 0.15 ^A	5.8 ± 1.3 ^B
4 Hypothyroid	4*	5	9.0 ± 0.3	95.3 ± 8.3 ^C	0.99 ± 0.07	10.7 ± 1.5 ^D
* indicates 2-days cold exposure only.						
5 Euthyroid	21	6	8.8 ± 0.7	24.2 ± 2.9	0.68 ± 0.18	2.1 ± 0.5
6 Hyperthyroid	21	6	9.6 ± 1.1	45.3 ± 5.2 ^E	0.55 ± 0.06	2.7 ± 0.5

Figure 3.5. Relative Activities of Mitochondrial GPAT and PPH using Activity Values from Table 3.6.

Figure 3.5a. Relative Activity of mitochondrial GPAT using Activity values from Table 3.6.

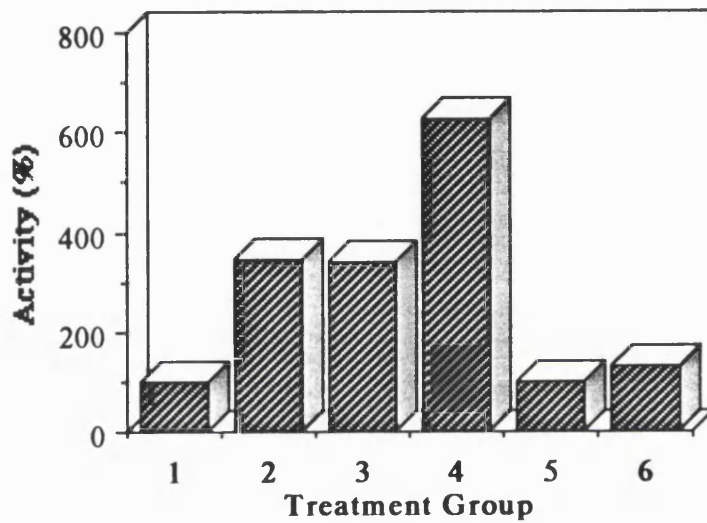
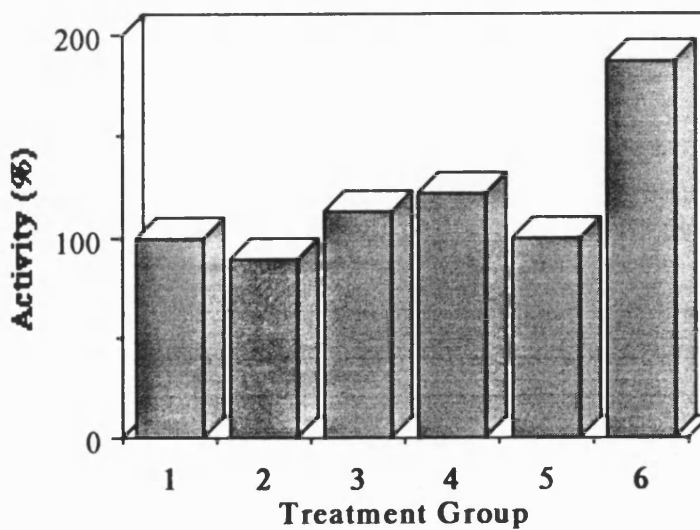


Figure 3.5b. Relative Activity of PPH using Activity values from Table 3.6.



(Table 3.5b).

GPAT and PPH catalyze the two most important steps in the glycerolipid synthesis pathway. GPAT is the first committed enzyme for the pathway and PPH directs glycerolipid synthesis towards triacylglycerol, PE and PC production. Both enzymes have been suggested to catalyze the rate-limiting step in the production of glycerolipids (see Introduction) and as such are of great interest. In BAT it has been established that the mitochondrial form of GPAT is the most responsive to physiological stimulation (cold-stress) of the tissue. Thus it was decided to concentrate further studies on the signals involved in regulation of the changes in activity of PPH and mitochondrial GPAT .

Table 3.6 and Figure 3.5a,b show the effect of thyroid status on the cold-induced changes in PPH and mitochondrial GPAT activities.

Hypothyroidism did not affect the activity of PPH at 21°C but caused a significant increase in the activity at 4°C. Cold-exposure of the euthyroid animals for 2 days did not significantly elevate PPH activity (Table 3.6). The activity of PPH has been demonstrated to double at 3 days cold-exposure (Table 3.2a) and Darnley *et. al.*, (1988) reported a significant increase in PPH activity at only 24 h. of cold-exposure. The lack of effect here is therefore a slightly anomalous observation and ought to be repeated.

Hypothyroidism in warm-adapted animals mimicked the 2-day cold-induced increase in mitochondrial GPAT activity (Table 3.6). Combination of cold-exposure and hypothyroidism resulted in a doubling of the effect of hypothyroidism or cold alone (Group 4, Table 3.6).

It appears that the effect of lack of thyroid hormone is to enhance the effect of cold in its stimulation of the activities of mitochondrial GPAT and PPH. This effect is in contrast to the lack of thermogenic activity in BAT from hypothyroid animals but agrees with the observations of Baht and Saggerson (1988) on the esterification potential of hypothyroid BAT. The increased production of triacylglycerol which could be expected to result from the changes recorded could explain the increased wet weight of BAT from hypothyroid animals.

Hypothyroidism has been demonstrated to result in a reduced sympathetic drive to BAT (Knehons and Romsos, 1984; Rothwell and Stock, 1984; Sundin, 1981) and as this results in the elevation of mitochondrial GPAT activity it provides more evidence that adrenergic pathways are not involved in the regulation of changes in glycerolipid synthesis in BAT. There is a significant reduction in the thermogenic capacity in hypothyroid BAT coupled with the doubling in the capacity of the esterification pathway (Blenneman *et. al.*, 1992) and the activity of the first committed enzyme in the pathway under the same conditions. This suggests that the signals controlling the two aspects of fatty acid utilisation are regulated via different signal pathways in BAT. The similarity in reduction in thermogenic capacity (Jamal and Saggerson, 1988; Woodward and Saggerson, 1989) but no reduction in the enzymatic capacity of glycerolipid synthesis between the hypothyroid and diabetic states provides more evidence for a link between the regulation of BAT metabolism in the two states.

Hyperthyroidism in warm-adapted animals did not significantly effect the activity of mitochondrial GPAT but doubled the activity of PPH (groups 5 and 6, Table 3.6). This apparently directly contrasts with the results obtained from the hypothyroid animals, as it now appears that both excess and lack of thyroid hormone are able to elevate PPH activity. However the period of hyperthyroidism studied was only 4 days compared to over 1 month of hypothyroidism. This period was sufficient to elevate GDP-binding to BAT mitochondria (Woodward and Saggerson, 1989), but it is possible that the results presented here only represent relatively short-term effects of hyperthyroidism and as such should not be directly compared to those of chronic hypothyroidism also presented.

3.2C. Effect of Adrenal Status on Cold-Acclimation Induced Changes in the Activity of Two of the Glycerolipid Synthesis Enzymes.

Previous studies with genetically obese rats and mice have indicated that adrenal hormones have an inhibitory effect on BAT thermogenesis and that adrenalectomy reverses this inhibition (Holt and York, 1982; 1984; Holt *et. al.*, 1983; Kim and Romsos, 1990; Marchington *et. al.*, 1983; Rothwell *et. al.*, 1984). Adrenalectomy results in increased

Table 3.7. Effect of Cold-Acclimation on Rat Weight and Interscapular Brown Adipose Tissue from Adrenalectomized Rats.

Table 3.7a. Body weights of rats during treatments and cold-exposure.

Treatment	Temp (°C)	n	Body wt. during treatment and/or cold-exposure	
			at start	at end
1 Sham-adrenalectomized	21	8	192 ± 5	222 ± 4 ^β
2 Sham-adrenalectomized	4	5	197 ± 4	189 ± 2
3 Adrenalectomized	21	4	184 ± 5	183 ± 6
4 Adrenalectomized	4	5	193 ± 2	172 ± 4 ^A

^{A,β}indicates $p < 0.02, 0.01$ respectively for the change in animal weight over the experimental period.

This legend also applies to Table 3.9a.

Table 3.7b. Details of interscapular adipose tissue from all groups studied.

For comparison of 2 versus 1, ^Aindicates $P < 0.001$. IV versus II, ^Bindicates $P < 0.001$.

Treatment	Temp (°C)	n	Interscapular brown-fat content of:		
			Wet wt. (g)	Protein (mg)	DNA (μg)
1 Sham-adrenalectomized	21	8	0.26 ± 0.02	13.9 ± 0.9	215 ± 17
2 Sham-adrenalectomized	4	5	0.26 ± 0.02	24.9 ± 1.7 ^A	294 ± 44
3 Adrenalectomized	21	4	0.29 ± 0.04	15.2 ± 0.9	167 ± 18
4 Adrenalectomized	4	5	0.21 ± 0.02	14.8 ± 1.0 ^B	227 ± 22

Table 3.8. Effects of cold-acclimation on mitochondrial GPAT and PPH activities in brown adipose tissue from adrenalectomised rats.

For comparison of 2-3 versus 1, ^{A,B}indicates $P < 0.001$, 0.01 respectively. 4 versus 2, ^{C,D} indicates $P < 0.05$, 0.001 respectively. 4 versus 3, ^Eindicates $P < 0.05$. In Group 1 $n = 4$ for the PPH values and $n = 8$ for the GPAT values.

Treatment	Temp (°C)	n	Phospatidate Phosphohydrolase activity as nmol per min:		Mitochondrial GPAT activity as nmol per min:	
			/mg of tissue protein	/100 µg of tissue DNA	/mg of tissue protein	/100 µg of tissue DNA
1 Sham-adrenalectomized	21	4/8	9.7 ± 0.9	68 ± 3.6	0.29 ± 0.03	1.9 ± 0.3
2 Sham-adrenalectomized	4	5	7.6 ± 0.7	70 ± 3.2	0.43 ± 0.03 ^B	4.0 ± 0.1 ^A
3 Adrenalectomized	21	4	11.0 ± 0.6 ^B	101 ± 4.1 ^A	0.56 ± 0.10	5.0 ± 0.6 ^A
4 Adrenalectomized	4	5	12.7 ± 0.6 ^D	84 ± 4.4 ^{C,E}	0.89 ± 0.13 ^D	6.1 ± 1.2

Figure 3.6. Relative Activities of Mitochondrial GPAT and PPH using Activity Values from Table 3.8.

Figure 3.6a. Relative Activity of mitochondrial GPAT using Activity values from Table 3.8.

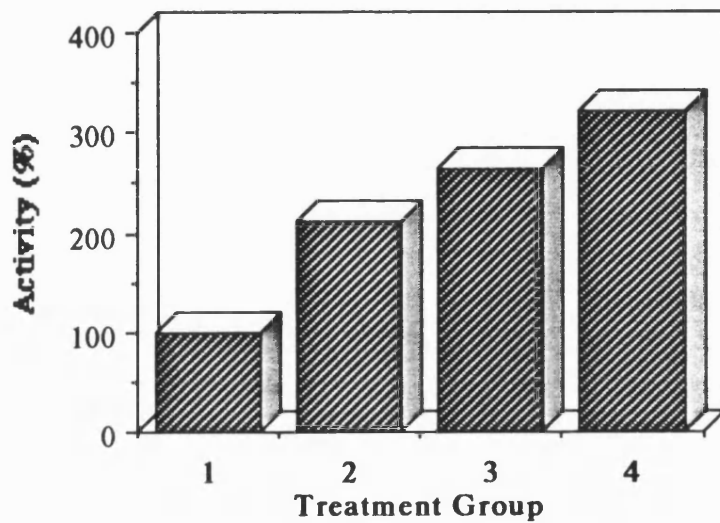
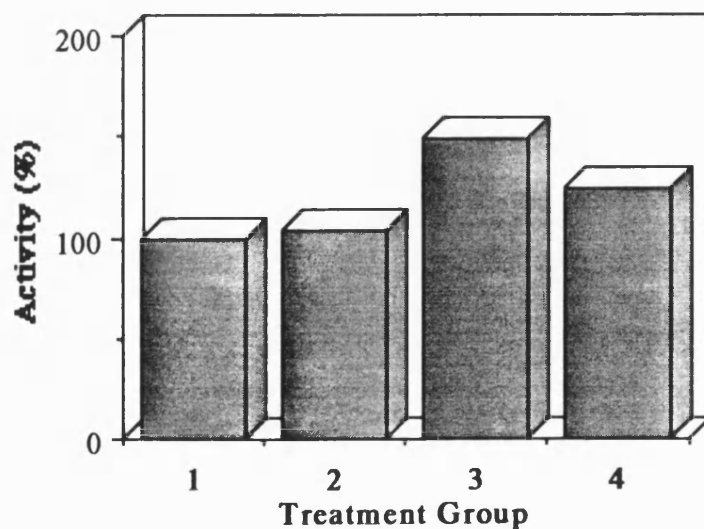


Figure 3.6b. Relative Activity of PPH using Activity values from Table 3.8.



synthesis and release of CRF which has been reported to have positive effects on BAT thermogenesis (Rothwell, 1989). Whether adrenalectomy has any significant effect in lean animals is unclear (Busbridge *et. al.*, 1990; Walker and Romsos, 1992).

In this study the effect of adrenalectomy itself and the effect on the cold-induced changes in mitochondrial GPAT and PPH activities was investigated.

Adrenalectomised rats were age-matched to sham-adrenalectomised controls for this study. Adrenalectomy, unlike hypothyroidism, did not affect survival of the animals at 4°C, so the period of cold-exposure could be that at which acclimation is initiated *i. e.* 3 days. The increase in rat weight seen in the sham-adrenalectomised animals was abolished by adrenalectomy and adrenalectomy enhanced the weight-loss during the 3 days cold-exposure. Adrenalectomy had no effect on BAT wet weight, total tissue protein or total tissue DNA and did not prevent the increase in total protein due to 3 days cold-exposure (Table 3.7a,b).

Table 3.8 and Figure 3.6a,b show the effect of adrenalectomy on the activity of mitochondrial GPAT and PPH in BAT from warm-adapted and cold-exposed rats. Both enzyme activities are elevated following adrenalectomy. The increase in mitochondrial GPAT activity after adrenalectomy mimics that induced by cold-exposure and does not enhance the effect of cold. In this experiment PPH activity was not elevated by cold-exposure in the sham-adrenalectomised animals. Adrenalectomy significantly elevated the enzyme activity but the combination of adrenalectomy and cold appeared to significantly decrease the activity of PPH (Table 3.8). Whether the apparent inhibition of cold-induction of PPH activity (Table 3.2a shows the normal induction of PPH activity by cold-exposure for 3 days) by operational stress is a valid physiological observation is not clear. The effects of adrenalectomy on PPH activity are conflicting and very difficult to rationalize at this time.

From the above results it would appear that removal of the adrenal gland is sufficient to mimic the cold-induction of mitochondrial GPAT activity. This suggests that the inhibitory role of the adrenal hormones on BAT activity in obese animals may also be

apparent in lean animals when considering the glycerolipid synthesis pathway enzymes in this tissue.

There is an increased synthesis and release of CRF in adrenalectomised animals which may result in an acute activation of sympathetic stimulation of BAT (Egawa *et al.*, 1990; LeFruvre *et al.*, 1987; Walker and Romsos, 1992). However it has been demonstrated that elevation of glycerolipid synthesis enzyme activities is not via an adrenergic mechanism (Tables 3.2 and 3.4). It is therefore unlikely that the elevation of PPH and mitochondrial GPAT at 21°C could be mediated by CRF-induced increased neural stimulation of BAT. It is possible that a reduction in adrenal hormones is sufficient to cause the effects on the enzyme activities both in warm-adapted and cold-exposed animals. However if this were the case then a physiological reduction in adrenal hormone levels due to cold exposure would have to occur. This has yet to be investigated.

3.3. Effect of Cycloheximide or Actinomycin D on the Cold-Induced Changes in Mitochondrial GPAT and PPH Activities in Brown Adipose Tissue.

The variations in PPH activity and the large variations in mitochondrial GPAT activity (a rough estimation of this variation is approximately 35-fold between the lower extreme of diabetes at 21°C and the higher extreme of hypothyroidism at 4°C) could be due to altered synthesis and turnover of enzyme protein, modification of existing enzyme or a combination of these two mechanisms. Therefore the effect of the protein synthesis inhibitors cycloheximide (inhibits at the level of translation) and actinomycin D (inhibits at the level of transcription) on the cold-response of mitochondrial GPAT and PPH was investigated.

Only a single dose of the drug was given immediately prior to the initiation of cold-exposure as previous attempts at daily boosters of the drugs resulted in death of the drug-treated animals. Even with this regime only 2 out of 6 of the actinomycin D treated cold-exposed group survived. Repeated attempts to improve this number failed.

Cycloheximide reduced the weight gain in warm-adapted and cold exposed animals. Cycloheximide also prevented the cold-induced increase in BAT total tissue protein seen in

Table 3.9. Effect of Cycloheximide or Actinomycin D on Changes in Rat Weights and Interscapular Brown Adipose Tissue from Cold-Acclimated Normal Rats.

Table 3.9a. Body weights of rats during treatments and cold-exposure.

Treatment	Temp (°C)	n	Body wt. during treatment and/or cold-exposure	
			at start	at end
1 None	21	6	180 ± 3	208 ± 3 ^B
2 None	4	6	184 ± 2	198 ± 2 ^B
3 Cycloheximide	21	6	179 ± 4	189 ± 4
4 Cycloheximide	4	5	181 ± 3	173 ± 3
5 None	21	6	205 ± 5	224 ± 5
6 None	4	6	191 ± 4	195 ± 5
7 Actinomycin D	21	4	197 ± 3	220 ± 4 ^B
8 Actinomycin D	4	2	191 ± 6	194 ± 15

Table 3.9b. Details of interscapular adipose tissues from all groups studied.

For comparison of 2 versus 1 and 6 versus 5, A, B indicates $P < 0.05$, 0.01 respectively. 4 versus 2, C indicates $P < 0.01$. IV versus III, D indicates $P < 0.05$.

Treatment	Temp (°C)	n	Interscapular brown-fat content of:		
			Wet wt. (g)	Protein (mg)	DNA (µg)
1 None	21	6	0.23 ± 0.02	11.9 ± 1.2	324 ± 25
2 None	4	6	0.24 ± 0.01	20.1 ± 2.2 ^B	403 ± 23 ^A
3 Cycloheximide	21	6	0.19 ± 0.02	11.6 ± 1.7	319 ± 51
4 Cycloheximide	4	5	0.19 ± 0.01 ^C	15.0 ± 1.8	459 ± 28 ^D
5 None	21	6	0.29 ± 0.03	11.6 ± 0.7	437 ± 77
6 None	4	6	0.27 ± 0.02	16.3 ± 1.1 ^B	574 ± 40
7 Actinomycin D	21	4	0.26 ± 0.03	13.0 ± 2.0	505 ± 45
8 Actinomycin D	4	2	0.26 ± 0.01	20.3 ± 1.4	310 ± 113

Table 3.10. Effect of Cycloheximide or Actinomycin D on Changes in Mitochondrial GPAT and PPH Activities in Brown Adipose Tissue from Cold-Acclimated Normal Rats

For comparison of 2-3 versus 1, A, B indicates $P < 0.05$, 0.01 respectively. 4 versus 2, C, D indicates $P < 0.05$, 0.02 respectively. 4 versus 3, E, F indicates $P < 0.01$, 0.001 respectively. 6 versus 5, G indicates $P < 0.001$. 8 versus 6, H indicates $P < 0.05$. VIII versus VII, I indicates $P < 0.02$.

Treatment	Temp (°C)	n	Phosphatidate Phosphohydrolase activity as nmol per min:		Mitochondrial GPAT activity as nmol per min:	
			/mg of tissue protein	/100 µg of tissue DNA	/mg of tissue protein	/100 µg of tissue DNA
1 None	21	6	12.5 ± 1.1	46 ± 5.3	0.16 ± 0.03	0.6 ± 0.1
2 None	4	6	9.3 ± 1.0	40 ± 4.4	0.44 ± 0.09 ^A	2.1 ± 0.4 ^B
3 Cycloheximide	21	6	10.6 ± 0.7	44 ± 1.9	0.08 ± 0.01 ^A	0.3 ± 0.06 ^A
4 Cycloheximide	4	5	9.4 ± 1.3	29 ± 1.8 ^{C, F}	0.24 ± 0.04 ^E	0.8 ± 0.1 ^{D, E}
5 None	21	6	12.7 ± 0.75	40.3 ± 8.5	0.30 ± 0.04	0.9 ± 0.2
6 None	4	6	9.9 ± 0.7	28.2 ± 1.4	1.05 ± 0.09 ^G	2.9 ± 0.1 ^G
7 Actinomycin D	21	4	13.3 ± 1.7	33.1 ± 4.0	0.32 ± 0.04	0.8 ± 0.2
8 Actinomycin D	4	2	10.7 ± 1.0	76.1 ± 16.6 ^H	1.39 ± 0.01 ^{H, J}	10.2 ± 3.0 ^I

Figure 3.7. Relative Activities of Mitochondrial GPAT and PPH using Activity Values from Table 3.10.

Figure 3.7a. Relative Activity of mitochondrial GPAT using Activity values from Table 3.10.

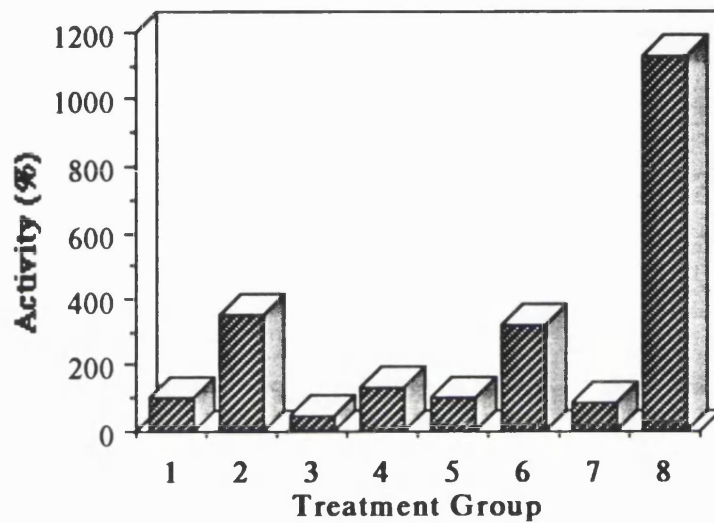
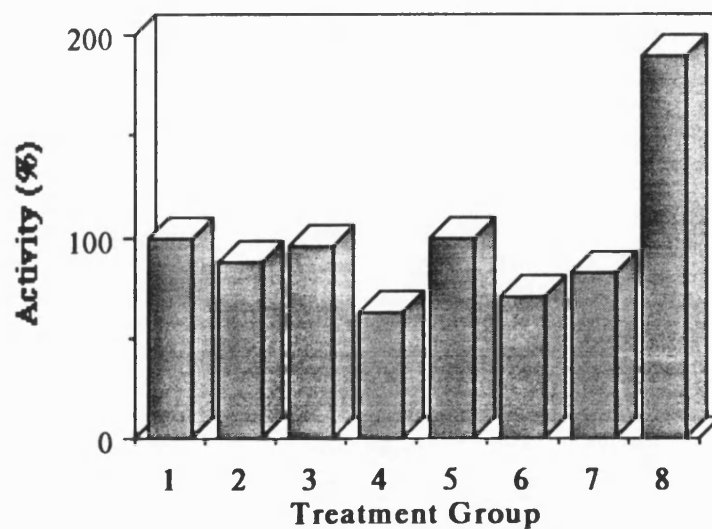


Figure 3.7b. Relative Activity of PPH using Activity values from Table 3.10.



untreated animals (Table 3.9a,b, groups 1-4).

Actinomycin D had no effect on weight gain but prevented the increase in total tissue protein at 4°C. However the loss of a significant increase in protein content may be due to the low number of surviving individuals in the actinomycin D treated, cold-exposed group (Table 3.9a,b, groups 5-8).

Table 3.10 and Figure 3.7a,b show the effect of either cycloheximide or actinomycin D on the cold-induced change in PPH and mitochondrial GPAT activities. Cycloheximide did not affect PPH activity in warm-adapted rats but significantly lowered the activity in the cold-exposed group. This suggested that the turnover rate of PPH may be elevated in the cold. Actinomycin D had no effect on PPH activity at 21°C but significantly elevated (by approximately 2-fold) the activity at 4°C.

Cold-exposure of untreated animals failed to show the increase in PPH activity seen previously (Table 3.2a, Darnley *et. al.*, 1988) in both the cycloheximide experimental set (Table 3.10, group 2) and the actinomycin D experimental groups (Table 3.10, group 6). Possible reasons for this lack of response could include simple animal variation, seasonal variation in the response to cold of PPH, and the fact that the time period in the cold is just at the threshold of the start of tissue hypertrophy.

Cycloheximide treatment at 21°C reduced the activity of mitochondrial GPAT by 50%, which could indicate, in a very crude manner, that the half-life of the enzyme is approximately 3 days at this temperature. The cold-induced increase in GPAT activity was reduced from 3.5-fold to 2.5-fold (or from 1.5 to 0.5 nmol/ min/100 µg DNA) by pretreatment with cycloheximide (Table 3.10, groups 1-4). As only one dose of cycloheximide was given it is possible that by the end of the experimental period some protein synthesis had resumed which may explain the small (but significant) increase in mitochondrial GPAT activity in the cold-exposed, cycloheximide treated animals. It is therefore possible to tentatively conclude that most, if not all, the cold-induced increase in mitochondrial GPAT activity is dependant upon protein synthesis. Actinomycin D did not

affect the activity of mitochondrial GPAT at 21°C but caused a 12-fold increase in activity at 4°C which was 4-fold higher than the effect of cold-exposure on untreated animals (Table 3.10, groups 5-8).

The extremely large increase in mitochondrial GPAT activity and the increase in PPH activity over and above the effect of cold at 4°C is an unusual and very interesting observation. However as this group of animals (group 8, Table 3.10) comprises only 2 individuals the results would require confirmation before being accepted as a "true" effect. However if the results were to be confirmed then a possible explanation of this drastic reaction to inhibition of transcription is available. If under normal cold-induction there is production of a short-lived negative regulator of the enzymes studied, this negative regulation would rapidly disappear in the presence of actinomycin D. This would result in an apparent increase of enzyme activity by the drug, providing that the half-life of the enzymes is not as short as that of the regulator. It must be stressed that the results and the above explanation are extremely speculative and would require a great deal of experimental work to confirm, which was not possible in the time available for this project.

One possible aid to elucidating whether the changes in enzyme activities are due to increased synthesis of protein or a modification of existing proteins is the use of antibodies to the enzymes. Using an ELISA system a specific measure of the levels of the proteins during the conditions and physiological states investigated above could then be made. Unfortunately no antibodies to mammalian GPAT or PPH exist at this time. This prompted an attempt to purify mitochondrial GPAT, from rat liver, with the aim of producing antibodies for use in an ELISA measurement of GPAT protein levels in BAT under the conditions investigated above.

3.4. Purification and Characterization of the Mitochondrial Form of Glycerolphosphate Acyltransferase from Rat Liver.

3.4A. Localisation of Glycerolphosphate within the Mitochondrion.

Previous reports (Daae and Bremer, 1970; Monroy *et. al.*, 1972; Nimmo, 1979) have indicated that mitochondrial GPAT resides exclusively in the outer mitochondrial membrane. However no measurement of cross contamination of membrane fractions and no statistical analysis of data presented was given. In some instances there was no data provided relating to marker enzymes included in the discussion of results. Thus these reports are less than convincing. Therefore it was decided to measure the activity of GPAT in both inner and outer liver mitochondrial membrane fractions from rats in different physiological states. The purity of the membrane fractions in terms of contamination with the second membrane (either inner in the outer fraction or *vice-versa*) was assessed using the activity of specific markers. The inner membrane marker used was succinate-dependant cytochrome *c*-reductase and zero activity of this enzyme was detectable in the outer membrane fractions used (not shown). The outer membrane marker used was rotenone-insensitive cytochrome *c*-reductase. The contamination of the inner membrane fraction by outer membrane could be calculated from the activity of this marker in the two fractions. These results are tabulated in Table 3.11. Also listed in Table 3.11. are the direct measurements of GPAT in both membrane fractions and the calculated outer membrane GPAT activity contaminating the inner membrane fraction. There would be no inner membrane contamination of the outer membrane fraction as no inner membrane marker was detected in this fraction. Thus, to see if the GPAT activity exclusive to the inner membrane within the inner membrane fraction was significant, a paired test of difference between the directly-measured inner membrane fraction GPAT activity and the activity due to contamination of the fraction with outer membrane, was carried-out. Table 3.11. shows that in each of the physiological states investigated, namely normal, starved and diabetic animals, there was a significant amount of GPAT activity which could not be attributed to the outer mitochondrial membrane. Figure 3.8. shows the GPAT activity associated with outer membranes compared to that associated with the inner membrane. An interesting point to note is that the ratio of inner to outer activity changes in starved and diabetic animals compared to the normal data. Whether this has any physiological significance,

Table 3.11. Presence of Significant Mitochondrial GPAT activity in Both Inner and Outer Membranes of Liver Mitochondria.

Animal status	specific activity outer membrane marker in outer membrane fraction (X)	specific activity outer membrane marker in inner membrane fraction (Y)	measured specific activity of GPAT in outer membrane fraction (Z)	measured specific activity of GPAT in inner membrane fraction	calculated specific activity of outer membrane GPAT in inner membrane fraction (Z x Y/X)
Normal (n = 6)	1572 ± 101	423 ± 28	3.3 ± 0.7	1.3 ± 0.13	0.88 ± 0.18 ^A
Starved 48 h. (n = 5)	1796 ± 158	273 ± 14	3.6 ± 0.5	1.3 ± 0.13	0.54 ± 0.06 ^B
Diabetic (n = 4)	990 ± 95	247 ± 28	2.7 ± 0.3	1.7 ± 0.13	0.77 ± 0.10 ^A

Table 3.11. Specific activities given in table 3.11 are all nmol/ min/ mg protein. The outer membrane marker enzyme used to give values X (from outer membrane fraction) and Y (from inner membrane fraction) was rotenone insensitive cytochrome *c*-reductase (see materials and methods for details of assay). The contamination of inner membranes with outer membranes was calculated and the specific activity of GPAT in the inner membrane due to outer membrane contamination given above (Z x Y/X values). Paired test of differences between total inner membrane GPAT activity and outer membrane contamination GPAT activity was performed and ^{A, B} indicates *p* < 0.025 and 0.01 respectively.

Figure 3.8. GPAT Activities in Inner and Outer Mitochondrial Membranes in Liver from Normal, Starved and Diabetic Rats.

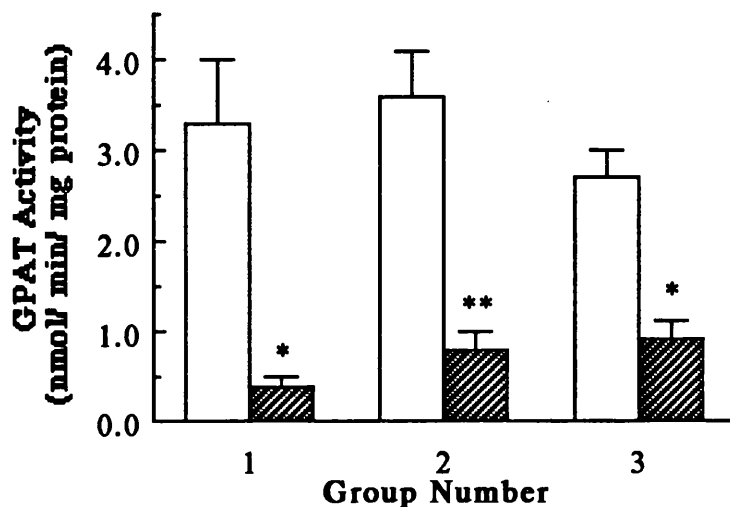


Figure 3.8. GPAT Activities in Inner and Outer Mitochondrial Membranes in liver from Normal, Starved and Diabetic Rats.

All assays contained bovine albumin (2 mg/ml), 10 mM NEM, 40 μ M palmitoyl-CoA and 1 mM [¹⁴C]glycerol phosphate. Details of the calculation of activities are given in the text.

Open bars, directly measured outer membrane activity

Hatched bars, calculated inner membrane activity. *,** indicate that these values are significantly different from zero ($P < 0.025$, < 0.01 respectively for a paired test).

Group 1 = normal fed animals (n = 6 membrane preparations); Group 2 = 48h-starved animals (n = 6 membrane preparations); Group 3 = diabetic animals (n = 4 membrane preparations).

remembering that GPAT activity in liver is decreased in both starvation and diabetes (see Introduction), is not clear and would require further study.

3.4B. Attempted Purification and Characterization of Mitochondrial GPAT from Rat Liver.

As it had been demonstrated (above results) that both inner and outer mitochondrial membranes in liver contain significant GPAT activity, it was decided to use total liver mitochondrial membranes as a starting point for the attempted purification of mitochondrial GPAT.

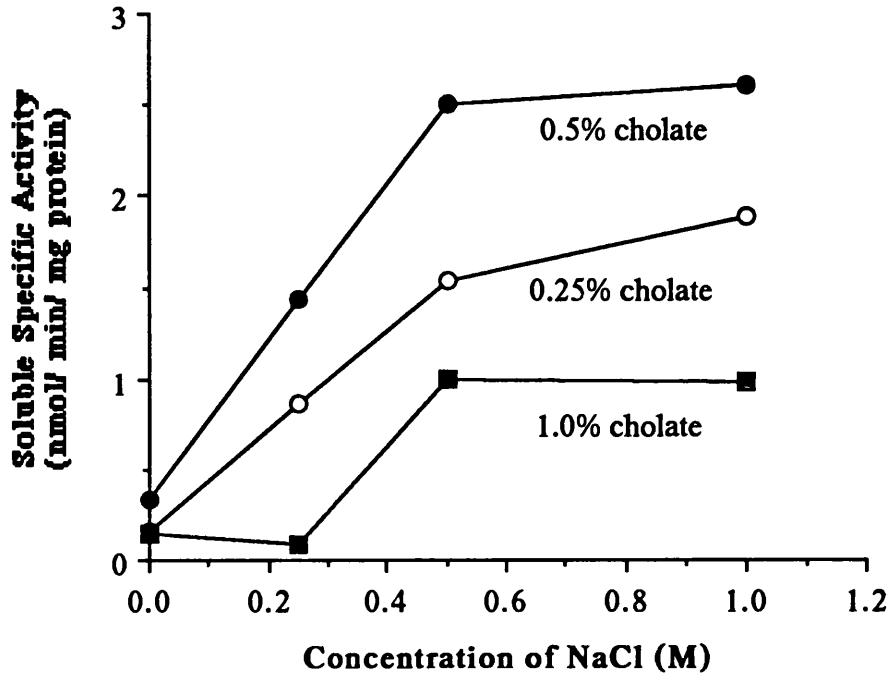
As stated in the Materials and Methods section, the solubilisation conditions chosen were adapted from previous attempts at purifying mitochondrial GPAT (Monroy *et. al.*, 1973; Mok and McMurry, 1990). The more recent of the attempts at purification has yielded upto a 40-fold increase in enzyme purity with an extremely low recovery of activity (Mok and McMurry, 1990). As Monroy *et. al.* (1973) were able to solubilise and stabilise 70% of their initial activity, it was decided to try to repeat and improve the method used by these authors. Figure 3.9a, b, shows that the optimum conditions for solubilisation of mitochondrial membranes, with maximal retention of GPAT activity, was 0.5% cholate in the presence of 1 M salt (either NaCl or KCl). These conditions were identical to those suggested by Monroy *et. al.* (1973), however there was no requirement for the presence of asolectin in the present study. Also an improvement of the method of Mok and McMurry was attempted but the only additional advantage achieved was to be able to solubilise membranes in any buffer and still retain over 50% of the initial GPAT activity, again in the absence of asolectin. Asolectin had been used in the previous studies to provide a mimic of the membrane environment from which the enzyme had been removed, as the compound is a mixture of phospholipids with phosphatidylcholine being the largest component. In this study addition of asolectin either at the assay stage or in any buffers used during chromatography failed to alter the results obtained. The solubilised form of GPAT was stabilised in solution by immediate removal of detergent and by bringing the buffer containing the enzyme activity to 20% glycerol. Stored in this manner at -70°C, the enzyme

Fig. 3.9. Optimisation of Conditions For the Solubilisation of Mitochondrial Membranes to Yield GPAT Activity in the Soluble Fraction.

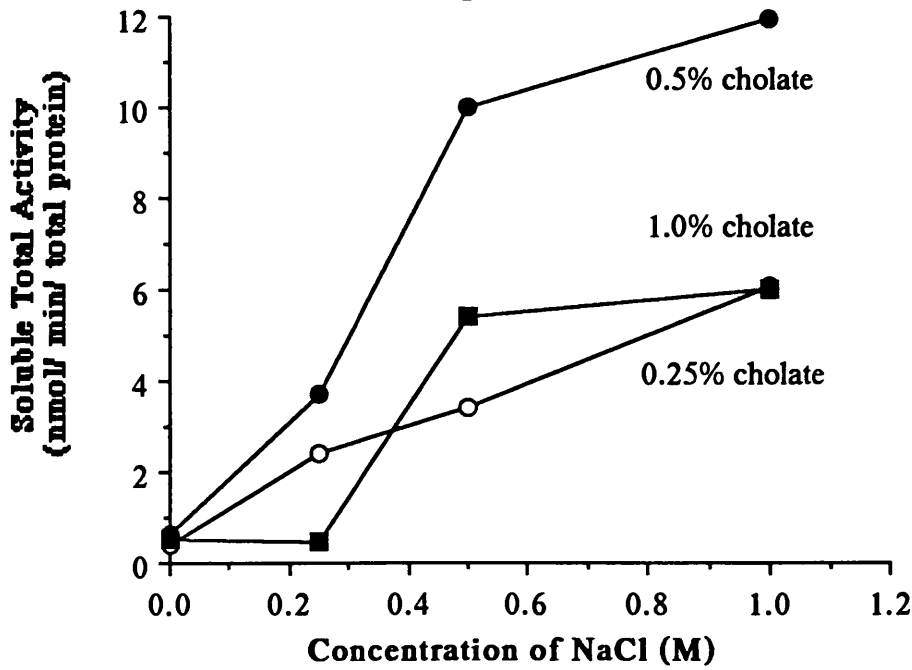
The concentration of NaCl was varied at different levels of the detergent (cholate). The mixtures were mixed on ice for 10 min. and then centrifuged at 105 000g for 1 h. Samples of the resultant supernatant were used to initiate assays of GPAT under the following conditions; 6 mg/ml BSA, 120 mM KCl, 1 mM DTT, 1 mM [¹⁴C-] glycerol phosphate, 40 μM palmitoyl-CoA and 100 mM Tris-HCl, pH 7.4. Figure 3.9a. shows GPAT specific activity in the soluble fraction and Figure 3.9b. shows GPAT total activity in the soluble fraction.

Optimal conditions for solubilisation of GPAT activity from mitochondrial membranes is shown to be 10 min. on ice, with 0.5% cholate at 1 M NaCl.

Effect of varying [NaCl] at different % cholate on solubilised GPAT activity.
Figure 3.9a.



Effect of varying [NaCl] at different % cholate on soluble GPAT activity.
Figure 3.9b



activity was stable for over 1 year, providing excessive freeze-thawing was avoided.

The first step in the purification of an enzyme is often to differentially precipitate protein using either ammonium sulphate or polyethylene glycol (PEG). This treatment can several have advantages; 1) enzymes are often much more stable in concentrated solutions or even in the precipitated form, 2) it is sometimes possible to achieve a degree of purification by careful differential precipitation of proteins across a range of final percentages of the precipitant, 3) the swapping of buffers can be rapidly achieved using this method. Therefore protein precipitation using either ammonium sulphate or PEG was carried-out on the soluble form of GPAT.

Ammonium sulphate precipitation was carried-out on solubilised samples containing GPAT activity. The salt was added in increments of 5% up to a final concentration of 50% ammonium sulphate. GPAT activity was progressively lost from the supernatant from 5% to 30% ammonium sulphate, however the activity was not recoverable in any resuspended pellet (Figure 3.10a). All pellets were resuspended in identical buffer to that in which solubilisation had taken place, but in the absence of any detergent. Removal of the salt by dialysis, or dilution and reconcentration, failed to improve recovery of GPAT activity in the ammonium sulphate precipitated pellets. The presence of asolectin also had no effect. The above observations could be interpreted as inhibition of GPAT activity by ammonium sulphate or inhibition of GPAT activity by precipitation.

A PEG precipitation study was carried-out in a similar manner to the ammonium sulphate study, with increments of 3% upto a final concentration of 30% PEG. Figure 3.10b shows that all the GPAT activity had been precipitated at 12-15% PEG and that this activity was recovered in the corresponding resuspended pellets. An approximate 2-fold increase in purity was obtained by this treatment and removal of the residual PEG was not necessary to maintain GPAT activity after resuspension. To obtain a solution of sufficient clarity, which could then be applied to a FPLC system for further purification, 0.1% cholate was added to the resuspension buffer. Unfortunately this led to an increase in the

Fig. 3.10. Precipitation of Solubilised GPAT Activity Using either Ammonium Sulphate or Polyethylene Glycol.

Increasing final percentages of either ammonium sulphate (Figure 3.10a.) or polyethylene Glycol (PEG) (Figure 3.10b.) were used to precipitate solubilised mitochondrial membrane proteins. The precipitated material was centrifuged away from material remaining in solution. After resuspension of pelleted material, aliquots of corresponding pelleted and non- pelleted material were used to initiate assays of GPAT. Assay conditions used were identical to those stated for Figure 3.9.

Only material pelleted by the action of PEG showed any GPAT activity, the highest activity being obtained at 12-15% PEG. An apparent 2-fold increase in the purity of the enzyme was also seen in this fraction, as the specific activity of the enzyme was doubled.

Figure 3.10a. Loss of Solubilised GPAT Activity Using Ammonium Sulphate.

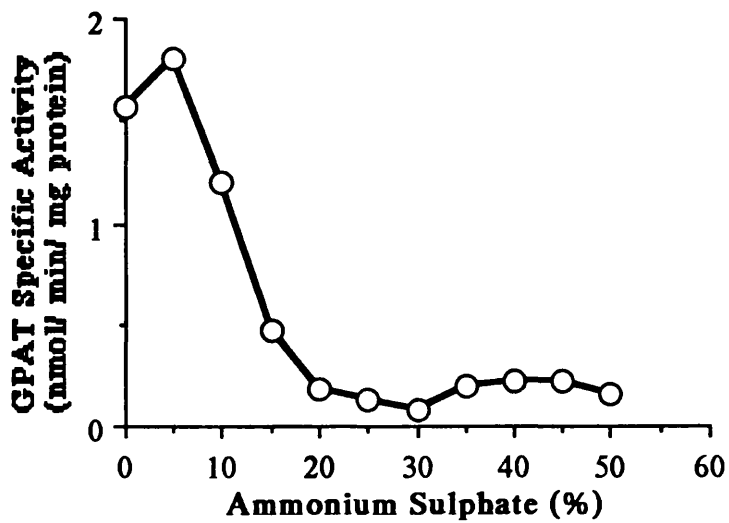
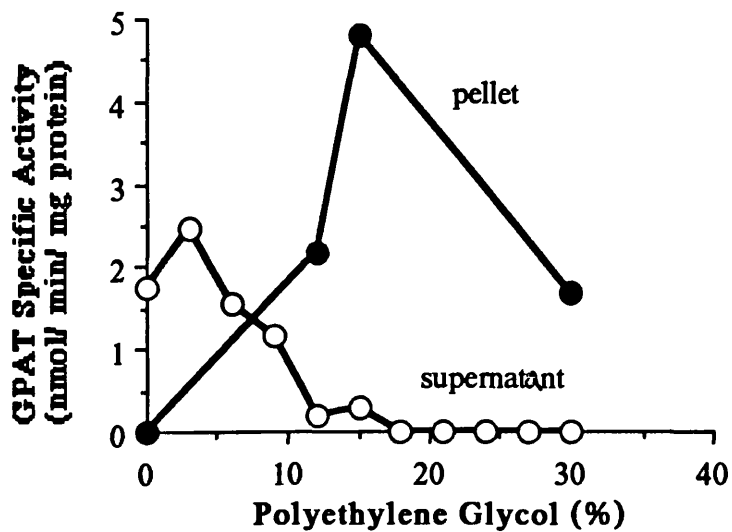


Figure 3.10b. Precipitation of Solubilised GPAT Activity Using Polyethylene Glycol.



rate at which the enzyme activity was lost during storage or further treatment. An alternative method for obtaining sufficient clarity of solution was to resuspend the protein pellet obtained in a very large volume of buffer (no detergent) and then concentrate the solution to improve enzyme stability. As glycerol was present in all buffers this process was slow and resulted in some loss of activity. Precipitation with 12-15% PEG occurred at too low a level of PEG for the process to be linked to hydrophobic interaction chromatography with any degree of success. The method did provide a way of exchanging the buffer in which the solubilised GPAT was suspended and in which the concentration of the solution (in terms of protein) could be regulated. Also the 2-fold increase in purity was an advantage. Unfortunately time did not allow development of the use of PEG precipitation in addition to other purification techniques.

Utilisation of protein characteristics such as hydrophobicity and overall surface charge is used to aid purification. Chromatography based on the above characteristics has been used extensively to purify many proteins and the development of the FPLC system has made these processes relatively rapid.

As GPAT is associated (a quite strong association is indicated by the fact that washing membranes with a high salt buffer did not release any activity) with mitochondrial membranes it is possible to assume that the protein may have exposed hydrophobic regions once it is removed from the membrane. Therefore hydrophobic interaction chromatography using a FPLC system, was attempted on cholate solubilised GPAT which had not been desalted prior to application to the column. The non-desalted sample was used because hydrophobic interactions are increased at high salt concentrations (1 M salt in this case). Elution from a hydrophobic column loaded at high salt is by decreasing salt concentration. The column media chosen was phenyl-sepharose as this is thought to have a more general interactive range than octyl-sepharose, the other, more specific, FPLC column matrix available. Unfortunately no binding to the column was observed and thus no increase in GPAT purity obtained. Increasing the salt concentration prior to application to the column to try to increase the hydrophobicity of the sample resulted in loss of GPAT activity (results

not shown), as did lowering the pH of the sample (also thought to increase hydrophobicity). The presence of any residual detergent may have interfered with interaction of protein with the column. It is possible that solubilisation of the mitochondrial membranes yielded proteins which were not totally free from surrounding phospholipids. This would also interfere with hydrophobic interactions by masking hydrophobic sites on the protein surface.

Ion-exchange chromatography separates proteins by utilising differences in the overall surface charge on proteins. An ion-exchange chromatographic medium was used in a previous attempt at purifying GPAT (Mok and McMurry, 1990). As the speed of purification was thought to be of importance, ion-exchange chromatography was performed using the FPLC system. The previous ion-exchange matrix used (DEAE-sepharose, Mok and McMurry, 1990) was weakly anionic, therefore it was decided to use the MONO-Q column available for the FPLC. MONO-Q (monobeads with a quaternary amine group as the functional group) is a strong anionic-exchange medium. As cholate, being an anionic detergent, would have bound irreversibly to the matrix, sample solubilised with Triton X-100 was applied to this column. Although protein bound to the column, and no GPAT activity was detected in the protein which washed through the column, elution with a linear gradient of 0-1 M NaCl, at pH 7.4, yielded no GPAT activity. Thus all the enzyme activity was lost. As surface charge can be affected by pH, the above was repeated at various pH values with the same result (although protein binding to the column was altered). It was thought that interaction of GPAT with the column matrix may be causing the observed total loss of activity. Therefore the solubilised sample was applied to a cationic-exchange column, MONO-S (functional group being CH_2SO_3). The idea was to try to bind other proteins to the matrix and separate GPAT from bound material by allowing this protein to wash through the column without binding to the matrix. Unfortunately no activity was recoverable either in the unbound material or the eluted proteins at any of the pH values tried.

A final attempt was made to try to purify GPAT using the extremely powerful

technique of affinity chromatography. This technique relies on proteins binding to a ligand which resembles either a substrate or an inhibitor or a natural ligand for the protein in question. Palmitoyl-CoA bound to agarose was used as an affinity matrix as palmitoyl-CoA is one of the substrates used in the reaction catalysed by GPAT. It would be expected that a good affinity between this matrix and GPAT would be seen, however no binding of GPAT activity to the matrix was observable. Addition of glycerol-3-phosphate to the buffer resulted in approximately 50% binding of GPAT activity to the matrix, measured using a batch-wise system. Palmitoyl-CoA agarose was suspended in the appropriate buffer and mixed with an equal volume of the same buffer containing GPAT activity and left on ice for 30 min., with frequent mild mixing. 50% loss of activity from the supernatant after pelleting of the affinity matrix (by centrifugation in a microfuge at full speed for 2 min.) was observed. This could indicate that the catalytic mechanism employed by GPAT *in vivo* requires binding of glycerol-3-phosphate prior to that of palmitoyl-CoA, the former inducing a conformational change in the active site of the enzyme allowing binding of the second substrate. Extensive further work would be required to confirm or refute this idea. Elution of protein bound to the affinity matrix, using 1 M KCl, did not yield any GPAT activity, *i. e.* bound GPAT activity could not be recovered.

Binding of protein to a more general affinity matrix, Blue-sepharose, was attempted using a batch-wise system. Blue-sepharose matrix was suspended in the appropriate buffer and mixed with an equal volume of the same buffer containing GPAT activity and left on ice for 30 min., with frequent mild mixing. This resulted in loss of GPAT activity from the buffer after pelleting of the affinity matrix (see above for conditions), with no extra additions to solubilisation buffers required. Elution of bound protein (bound protein presumably including GPAT) from the affinity matrix by mixing with buffer containing high salt and/ or 10 mM NADH, and separating the matrix from the buffer as described above, failed to recover any GPAT activity. This total loss of GPAT activity was seen in all chromatographic treatments reported in the present study.

Further procedures which could be investigated in an attempt to purify an active

form of GPAT include; 1) Development of a coloured activity stain specific for GPAT which could be used in conjunction with a native gel. This would allow the area on the gel containing GPAT activity to be identified and then electroeluted into an appropriate buffer and purity checked.

2) A re-assessment of the solubilisation of mitochondrial membranes could be made with the idea of utilising a detergent which would not need to be removed to maintain activity. This would eliminate the possibility that activity is lost as purity is increased due to aggregation of GPAT masking active sites on the protein.

3) The chromatography studies could be repeated with one or both substrates present in all buffers used, as this is known to stabilise some proteins during purification.

4) If antibodies to the purified *E. coli*. GPAT were to become available, studies could be carried-out into the possibility of cross-reactivity between the antibody and the enzyme derived from the two species. Should cross-reactivity be seen then a matrix bound to the *E. coli*.-GPAT antibodies would provide a very powerful and rapid tool which could be used to purify active mammalian mitochondrial GPAT.

The inability to be able to retain GPAT activity during any of the treatments attempted and reported above was both frustrating and time consuming. Although many other ways in which to attempt purification of GPAT can be envisaged (see above) it was decided to investigate whether solubilisation of the membrane bound mitochondrial GPAT changed the characteristics of the enzyme. The reasoning for this is that in the cell GPAT exists in two forms; mitochondrial and microsomal GPAT. Each form of the enzyme has different characteristics, for example acyl-CoA substrate specificity and susceptibility to inhibition by thiol-group reagents, such as N-ethylmaleimide (NEM). It is possible that these differences are due only to the membrane environment and that the core enzyme is identical. Hence removal of the mitochondrial enzyme from its membrane may cause the enzyme to behave in a manner very similar to that of the microsomal enzyme, which would indicate that the two forms of GPAT are simply ^{the same} enzyme in different environments.

Solubilisation of mitochondrial GPAT resulted in a change in the optimum

Figure 3.11. Effect of Solubilisation on Dependence of Mitochondrial GPAT on Palmitoyl-CoA Concentration.

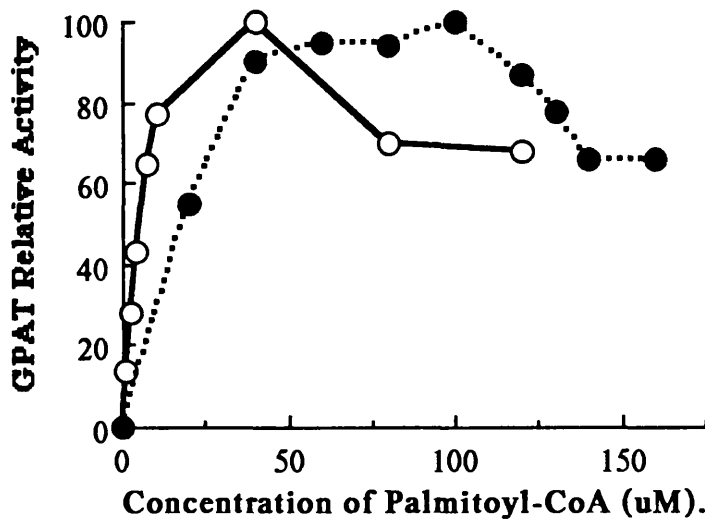


Fig.3.11. Effect of solubilization on dependence of mitochondrial GPAT on palmitoyl-CoA concentration

All assays contained bovine albumin (6 mg/ml), 1 mM DTT, 1 mM [¹⁴C]glycerol phosphate and the indicated concentrations of palmitoyl-CoA

●, GPAT *in situ* in total mitochondrial membranes

○, GPAT solubilized from total mitochondrial membranes.

concentration of palmitoyl-CoA needed for maximal activity. Figure 3.11 shows that, using the comparative assay conditions stated in Materials and Methods (6 mg/ml fatty-acid free albumin, 120 mM KCl, 1 mM DTT and 100 mM Tris-HCl, pH 7.4), the palmitoyl-CoA optimum concentration for the membrane bound form of mitochondrial GPAT was 100 μ M compared to 40 μ M for the soluble form.

The K_m for glycerol-3-phosphate, at optimal palmitoyl-CoA concentrations, was also different for the membrane bound and soluble forms of mitochondrial GPAT. Figure 3.12a, b shows the substrate concentration versus activity curve for glycerol-3-phosphate (Figure 3.12a) and an Eadie-Hofstee transformation of the data in Figure 3.12a (Figure 3.12b). From the graphs shown the K_m and V_{max} for the two forms of the enzyme can be determined. The K_m is altered from the membrane bound enzyme value of 2.5 mM to the soluble enzyme value of 5.8 mM. The V_{max} of the enzyme, was 9.9 nmol/min/mg protein and 14.1 nmol/min/mg protein for membrane bound and soluble activities respectively. Whether the change in the K_m of the enzyme has any physiological significance is unclear at this time.

The acyl-CoA specificity of the soluble mitochondrial GPAT was compared to its membrane bound 'parent' and to the microsomal form of the enzyme. Figure 3.13a, b, c demonstrates that removal of the mitochondrial enzyme from its membrane did not alter its acyl-CoA specificity. Both membrane bound and soluble mitochondrial GPAT only utilised palmitoyl-CoA whereas microsomal GPAT was able to use both palmitoyl-CoA and oleoyl-CoA equally well.

The most striking difference between the microsomal and mitochondrial forms of GPAT is the susceptibility to inhibition by thiol-group reagents. Microsomal GPAT activity is totally inhibited in the presence of 10 mM NEM, but mitochondrial GPAT activity is unaffected. Figure 3.14 shows that solubilisation of the mitochondrial GPAT away from the membrane results in an increase in the ability of NEM (the thiol-group reagent used) to inhibit enzyme activity. Soluble mitochondrial GPAT is inhibited by approximately 65% by

Fig.3.12a, b. Effect of solubilization on the dependence of mitochondrial GPAT on glycerol phosphate concentration

All assays contained bovine albumin (6 mg/ml), 1 mM DTT, [¹⁴C]glycerol phosphate (0.1 - 5 mM) and optimum concentrations of palmitoyl-CoA which were 100 μM and 40 μM for membrane-bound and solubilized GPAT respectively (see Fig.3.11.). In figure 3.12b the values are shown as an Eadie-Hofstee plot.

O, GPAT *in situ* in total mitochondrial membranes ($V_{\max} = 9.9 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg of protein}^{-1}$, $K_m = 2.5 \text{ mM}$)

▲, GPAT solubilized from total mitochondrial membranes ($V_{\max} = 14.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg of protein}^{-1}$, $K_m = 5.8 \text{ mM}$).

Figure 3.12a. Effect of solubilization on the dependence of mitochondrial GPAT on glycerol phosphate concentration.

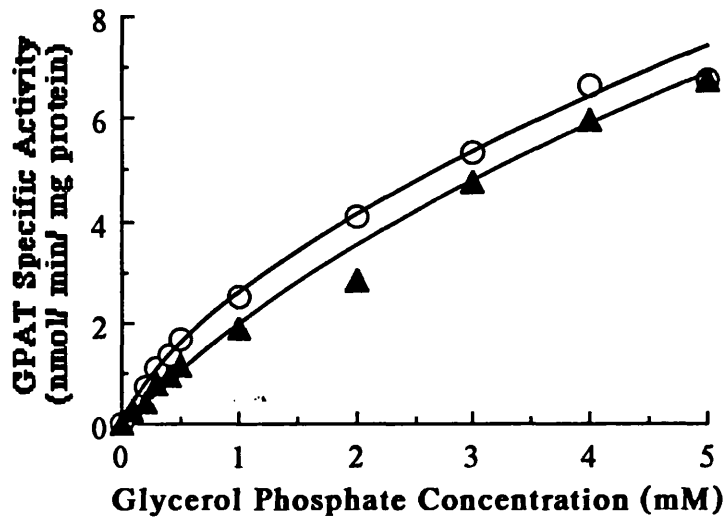


Figure 3.12b. Eadie-Hofstee Plot of Using Values from Figure 3.12a.

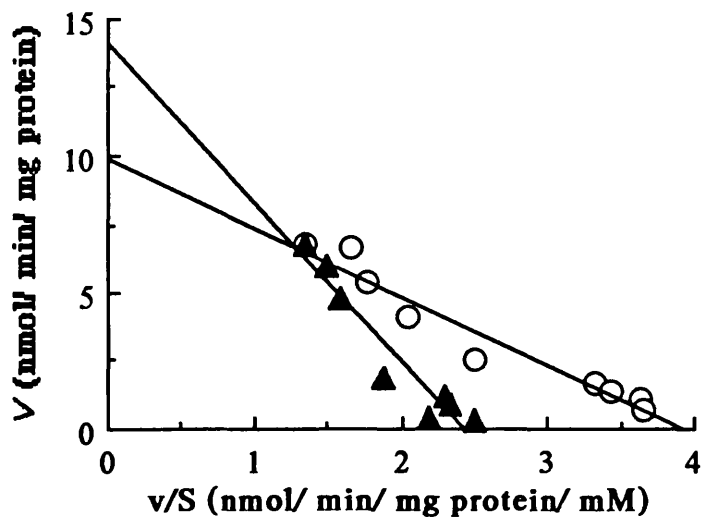


Fig.3.13. Acyl-group specificity of GPAT preparations

All assays contained bovine albumin (6 mg/ml), 1 mM DTT, 1 mM [¹⁴C]glycerol phosphate and the indicated concentrations of palmitoyl-CoA (○) or oleoyl-CoA (●).

- (a) GPAT *in situ* in total mitochondrial membranes
- (b) GPAT solubilized from total mitochondrial membranes
- (c) GPAT *in situ* in microsomal membranes.

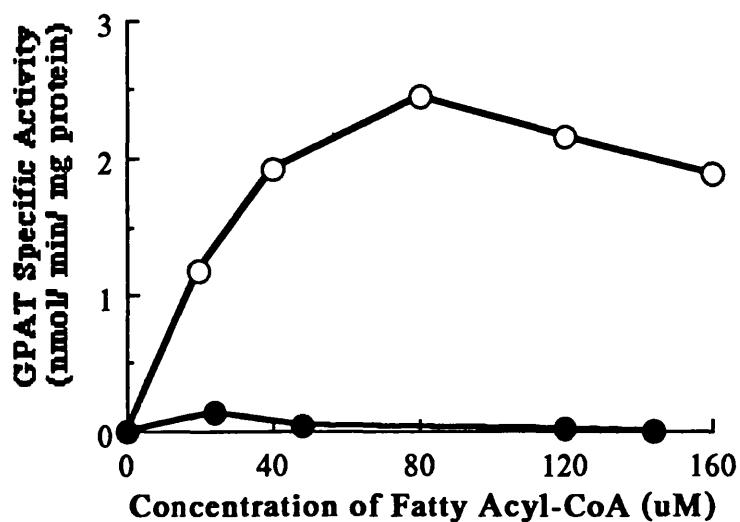
Figure 3.13a. Acyl-group Specificity of GPAT *in situ* in Total Mitochondrial Membranes.

Figure 3.13b. Acyl-group Specificity of GPAT Solubilised from Total Mitochondrial Membranes.

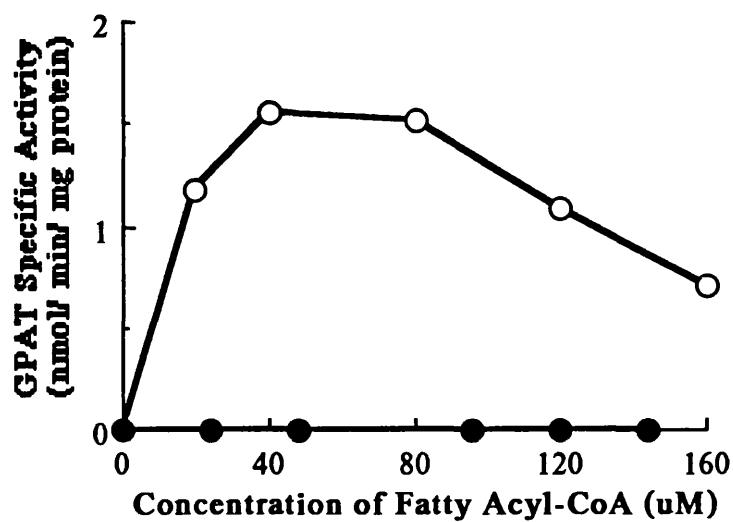


Figure 3.13c. Acyl-group Specificity of GPAT *in situ*. in Total Microsomal Membranes.

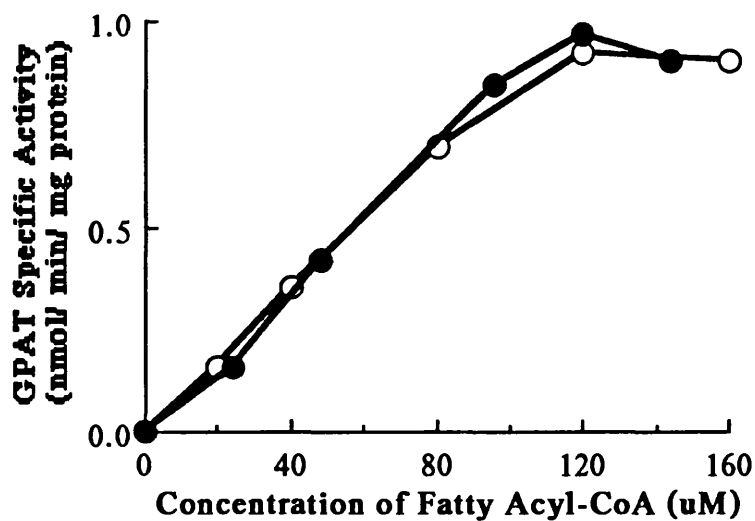


Fig 3.14. Inhibition of solubilized mitochondrial GPAT by N-ethylmaleimide

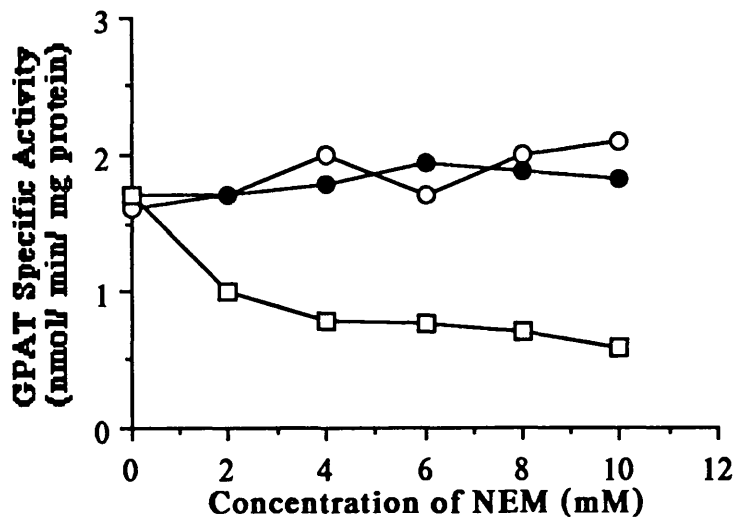
Preparations were preincubated for 5 min with the indicated concentrations of NEM before aliquots were taken to initiate GPAT assays. The assays contained bovine albumin (6 mg/ml), 1 mM [¹⁴C]glycerol phosphate and optimum concentrations of palmitoyl-CoA (see Fig.3.11). These were:

100 μ M for GPAT *in situ* in total mitochondrial membranes (○)

40 μ M for GPAT solubilized from total mitochondrial membranes without preincubation (●)

40 μ M for GPAT solubilized from total mitochondrial membranes (□).

Effect of NEM Presence on Mitochondrial GPAT Activity



preincubation for a short period with 10 mM NEM. This suggests that the mitochondrial membrane is responsible for protecting GPAT from inhibition by the action of thiol-group reagents. It also suggests that the difference between the two membrane bound enzymes *in vivo* in this aspect is likely to be due to their membrane environment and is not an intrinsic difference, as appears to be the case for their respective acyl-CoA specificities.

The above comparisons between mitochondrial GPAT in its membrane bound and soluble states with the microsomal GPAT appear to add evidence to the suggestion that the two forms of GPAT are different and might be regarded as isoforms of the same enzyme. However a more ideal set of comparisons, with a more detailed study of differences than time allowed here, would involve either a stable soluble form of both microsomal and mitochondrial enzymes or purified forms of the two forms of GPAT. Until this has been achieved the discussion over the nature of the two forms of GPAT in mammalian cells will not be resolved.

GENERAL DISCUSSION.

CHAPTER FOUR.

Previous research has demonstrated that cold-exposure for over 24 h. induces a significant increase in the activities of the glycerolipid synthesis enzymes in brown adipose tissue from normal rats (Darnley *et. al.*, 1988). The glycerolipid enzymes have been localized to the microsomal and soluble fractions of the cell, with the exception of long-chain fatty acyl-CoA synthase (FAS) and mitochondrial glycerolphosphate acyltransferase (GPAT). The esterification capacity of brown adipose tissue has been estimated to be equal to its oxidative capacity (Baht and Saggerson, 1988a). Treatment of brown adipocytes with noradrenaline increased glycerolipid synthesis (Baht and Saggerson, 1988b). Fasting inhibits brown adipose tissue (BAT) glycerolipid synthesis and can be overcome by refeeding (Mercer and Williamson, 1988).

This study has confirmed the observations of Darnley *et. al.* (1988), and for the first time investigated the hormonal regulation of glycerolipid synthesis in BAT. In this study the cold-induced changes in enzyme activities have been recorded at the threshold of BAT recruitment when mitochondriogenesis and tissue hypertrophy are just commencing *i. e.* 3-days cold-exposure. Thus the level of increases in enzyme activities recorded are variable over the same period of cold-exposure due to rapid changes in levels of tissue protein and total tissue DNA. The separate studies all show 'control' non-treated, warm-adapted groups. The activity values expressed as nmol/ min/ 100 μ g DNA are much more comparable as cellular DNA levels are at least constant between cells, unless they are in the process of division, even if tissue mass is changing.

The glycerolipid synthesis pathway provides both triacylglycerols for energy storage and phospholipids essential for membrane production. In BAT after 3-days cold exposure both products of the pathway would be required. Firstly to replenish lost triglyceride stores used in the initial burst of thermogenesis and secondly to provide phospholipids for the increased membrane (mitochondrial initially, followed by cellular) production initiated at this time. However the increase in thermogenesis also occurring would compete for the joint substrate for both esterification and β -oxidation, fatty acyl-CoA. Both processes are required to occur simultaneously in BAT and a fine balance

between the two pathways involved must be maintained. It would seem possible that the control point for the two processes could involve the enzyme catalyzing the formation of one of the substrates for both processes *i.e.* fatty acyl-CoA. Indeed the predominantly mitochondrial location of FAS in BAT indicates its important role in supplying fatty-acyl-CoA to the mitochondria for oxidation to produce heat when activated thermogenin has uncoupled BAT mitochondria. However the extent of thermogenesis due to BAT stimulation appears to be regulated directly at the level of thermogenin amount and activation (Himms-Hagen, 1989). Also there is only a two-fold increase in FAS activity due to 3-days cold-exposure (Darnley *et. al.*, 1988; and the results presented here). The regulation of that cold-induced increase appears to be via insulin-mediated pathways and does not appear to require adrenergic stimulation. This statement would require a further conformation as this study has only demonstrated that diabetes inhibits cold-induction of FAS and adrenergic stimulation in warm-adapted animals does not induce a mimic of the effect of cold-exposure. The possibility that both insulin and adrenergic stimulation are required to induce increased FAS activity in BAT, in a similar manner to the increase in thermogenin levels (Geleon and Trayhurn, 1990a; Yoshioka *et. al.*, 1989), has not been studied here and would form an ideal further experimental study. FAS in white adipose tissue also appears to require insulin for full activity as the enzyme activity is lowered in diabetes and fasting in this tissue (Saggerson and Carpenter, 1987). FAS in white adipose tissue is localized primarily in the microsomal fraction where all of the glycerolipid synthesis enzymes, except PPH activity which is also located in the soluble fraction and mitochondrial GPAT, are located. The mitochondrial form of GPAT has a much lower activity than its microsomal counterpart in white adipose tissue (Baht and Saggerson, 1988a). This could reflect the fact that in this tissue esterification is far in excess of oxidation thus supply of fatty acyl-CoA to the mitochondria would not be as important as is the case in BAT where FAS is predominantly located in the mitochondrial fraction (Baht and Saggerson, 1988a).

The first committed enzyme in the glycerolipid synthesis pathway is

glycerolphosphate acyltransferase (GPAT). The regulation of cold-induction of the mitochondrial form of GPAT in BAT will be discussed later. The activity of the microsomal form of GPAT is approximately doubled by 3-days cold-exposure and this increase is mimicked by β -adrenergic stimulation in warm-adapted animals. Diabetes lowered the basal level of activity by 40% but did not prevent the increase in activity due to exposure to cold. This strongly indicates that cold-induced changes in microsomal GPAT are regulated via the β -adrenergic mediated second messenger pathways, with a possible basal regulation of activity via the insulin mediated pathways. The apparent dual aspect of microsomal GPAT regulation adds support to the idea that both insulin and adrenergic stimulation act in concert to elicit the full response to stimulation of BAT. The effect of adrenergic stimulation on microsomal GPAT in BAT is in contrast to the inhibitory effects seen in white adipose tissue. However the role of insulin in regulating basal levels of activity in BAT corresponds to the similar decreased activity of microsomal GPAT in white adipose tissue in diabetes and fasting (Soorana and Saggerson, 1978). Although GPAT is an obvious choice as the regulatory enzyme of the pathway there is little evidence to suggest that the microsomal form of GPAT is that enzyme.

The next enzyme in the pathway is monoacyl-glycerolphosphate acyltransferase (MGPAT). The activity of MGPAT was only significantly increased by 3-days cold-exposure in BAT from diabetic animals and normal animals simultaneously treated with BRL-26830A. The physiological significance of these observations is difficult to assess since no other reports of hormonal regulation of MGPAT in other tissues exist. The apparent inhibitory effect of insulin on cold-induced increases in MGPAT activity is in contrast to the apparent insulin requirement for activity of FAS and microsomal GPAT. Baht and Saggerson (1988) have reported that, in brown adipocytes from warm-adapted rats, hypothyroidism induces a 2-fold increase in MGPAT activity. Clearly much further work on the hormonal signals involved in the regulation of this enzyme is required.

The enzyme catalyzing the conversion of phosphatidate to diacylglycerol, thus directing glycerolipid synthesis towards triacylglycerol, phosphatidylcholine and

phosphatidylethanolamine production, is Mg^{2+} -dependant phosphatidate phosphohydrolase (PPH). This enzyme occurs at an important branch point in the glycerolipid synthesis pathway and has been strongly suggested as the regulatory enzyme for the pathway in liver (Murthy and Schipp, 1979; Woods *et. al.*, 1981). Cold-exposure for 3-days initially induced a doubling in PPH activity in BAT from normal rats. This increase was not seen in BAT from diabetic animals and adrenergic stimulation did not mimic the cold effect. These observations suggest that the regulation of changes in PPH activity in BAT are very similar, if not identical, to that described for FAS.

In this study 2-days cold-exposure failed to significantly elevate PPH activity in BAT from 9 week old euthyroid rats. This observation is in contrast to that of Darnley *et. al.* (1988) who reported a significant increase in PPH from BAT at only 24 h. cold-exposure. Whether this difference is due to the 'threshold' effects in the tissue, increased age of the animals used in this study compared to Darnley *et. al.* (1988), or seasonal variations is not clear and a repeat study would be advisable. The role of the thyroid hormones in PPH regulation appears confused as hyperthyroidism elevates warm-adapted BAT PPH activity and hypothyroidism potentiates the effect of 2-days cold-exposure. The observation that hypothyroidism doubles the esterification potential of, and PPH activity in, brown adipocytes (Baht and Saggerson, 1988a) would support the observation of hypothyroid potentiation of cold-induction of PPH activity in BAT.

The adrenal hormones appear to have an inhibitory effect on PPH activity in BAT as adrenalectomy induces increased PPH activity which is not further elevated by cold-exposure. Sham-adrenalectomized cold-exposed animals did not show the increase in PPH activity seen previously in non-treated controls. This implies that operative stress inhibits up-regulation of PPH in BAT. However the apparent variability in the response of PPH activity to cold-exposure seen in other experiments should be considered and the non-response to cold seen in this study may be purely an example of that variability.

If the inhibition of PPH activity seen by combined α - and β -adrenergic stimulation was a true effect and not just an overload of the α -adrenergic agonist, then the effect of

sham-adrenalectomy could be explained as a long-term effect of elevated noradrenaline which would be associated with operative stress. However increased synthesis and release of CRF seen in adrenalectomized animals results in increased sympathetic drive to BAT (Egawa *et. al.*, 1990; LeFeuvre *et. al.*, 1987; Walker and Ramsos, 1992) which would suggest that noradrenaline levels would be high in the adrenalectomized animals as well. The elevated levels of PPH activity seen in these animals could then be larger than actually recorded, or the above explanation of the loss of the cold-induced effect in the sham-adrenalectomized animals could be incorrect. This would require further study, such as a direct comparison of non-operated controls with sham-adrenalectomized animals, to confirm. The inhibitory effect of adrenal hormones on thermogenesis in BAT has been noted previously (Holt *et. al.*, 1983; Freedman *et. al.*, 1986; York, 1989) but only in obese animal models.

The lack of a cold-induced increase in PPH activity in BAT was observed again in the animals used to investigate the effect of inhibition of protein synthesis on changes in enzyme activities. This in addition to the result seen with the sham-adrenalectomized animals suggests the possibility that the activity of PPH is not significantly elevated in BAT by cold-exposure for 3-days.

The cycloheximide experiment is difficult to assess due to the problem with the PPH activity cold-response, but the reduction in PPH activity in the cycloheximide-treated cold-exposed animals suggests that the stability of PPH in BAT may be reduced in the cold, *i. e.* PPH turnover rate may be increased in the cold. This increase in protein turnover is also seen in the case of thermogenin (Jacobson *et. al.*, 1987) in cold-stressed BAT.

Mitochondrial glycerolphosphate acyltransferase (GPAT) activity is elevated up to 8-fold above warm-adapted levels by cold-exposure for 3-days. This large increase is not mimicked by adrenergic stimulation and is not inhibited in diabetic rats. However diabetes does induce a significant reduction in basal mitochondrial GPAT levels, indicating a regulation of GPAT activity by insulin-mediated pathways in quiescent BAT. This was also seen with the microsomal form of GPAT. However the different regulation of the two

forms of GPAT in this tissue by the adrenergic mediated pathways and the fact that solubilisation of Liver mitochondrial GPAT did not alter the acyl-specificity of the enzyme, combine to indicate that the two cellular forms of GPAT are not ^{the same} an enzyme but distinct forms of GPAT.

Hyperthyroidism in warm-adapted animals had no effect on mitochondrial GPAT activity unlike the effect on PPH activity. Hypothyroidism resulted in a mimic of the 2-day cold-induced increase in mitochondrial GPAT activity and the effect of cold was further superimposed on GPAT activity in the hypothyroid state. The increase in GPAT activity in the hypothyroid state agrees remarkably well with the observations of the same conditions made in brown adipocytes (Baht and Saggerson, 1988a). The fact that cold-exposure was able to further elevate mitochondrial GPAT activity indicates that lack of thyroid hormone is not the signal responsible for the cold-induction of mitochondrial GPAT in BAT. Indeed the levels of thyroid hormones are elevated in cold-acclimated BAT but mitochondrial activity is still elevated by cold. It is possible to speculate that the elevation of the activity of mitochondrial GPAT in hypothyroid BAT is a means of directing any increased supply of fatty acids to the tissue, either by increased lipogenesis or supply from extracellular sources, into triacylglycerol stores. The increased triglyceride stores would give the quiescent BAT the appearance of white adipose tissue, a phenomenon often seen in BAT which is atrophied by lack of stimulation *e. g.* after denervation, when animals are returned to thermoneutrality and due to increasing age. The increase in the activity of mitochondrial GPAT during cold-exposure in the diabetic could function in a similar manner to that described for hypothyroidism, by channeling fatty acids supplied to the tissue into triacylglycerol while thermogenesis is inhibited. The insulin requirement for activity of GPAT is also observed in white adipose tissue but hypothyroidism results in decreased GPAT activity in this tissue (Taylor and Saggerson, 1986). It should be noted that the changes in GPAT activity in white adipose tissue are likely to be due to changes in the absolute activity of the microsomal form of the enzyme, even though the mitochondrial activity is also altered (Saggerson and Carpenter, 1987), as this is the predominant form in

the tissue. As we have demonstrated above that the two forms of GPAT are regulated differently in BAT this could explain the discrepancy between GPAT activity regulation in BAT and white adipose tissue in the hypothyroid state. Assay of the two forms of GPAT in BAT in all the conditions described could clarify this point.

Adrenalectomy resulted in increased mitochondrial GPAT activity in BAT from warm-adapted rats which mimicked the effect of 3-days cold-exposure. Cold-exposure of the adrenalectomized rats did not further elevate the activity of mitochondrial GPAT in BAT. These results suggest that the cold-induction of mitochondrial GPAT is mediated by a reduction in levels of adrenal hormones in or around BAT. The increased sympathetic stimulation of BAT observed in adrenalectomized animals is not likely to cause the increase in mitochondrial GPAT activity since I have demonstrated that α - or β -adrenergic stimulation, or a combination of both, is unable to elevate mitochondrial GPAT activity in BAT from warm-adapted or cold-exposed rats. An ideal further experimental study arising from these observation would be to measure the adrenal hormone levels in and around BAT from warm-adapted and cold-exposed rats. Also administration of adrenal hormones to adrenalectomized animals, and their sham-adrenalectomized controls, prior to and during cold-exposure would provide direct evidence of the effect of the adrenal hormones on this enzyme. Obviously assessment of the changes in the activities of the other enzymes in the glycerolipid synthesis pathway would also be of interest and could provide a more complete picture of the regulation of the whole pathway in brown adipose tissue.

From the studies with cycloheximide it would appear that a rough, indirect, measurement of the half-life ($t_{1/2}$) of mitochondrial GPAT gives a value of approximately 3 days. This is because the activity of mitochondrial GPAT is reduced by 50% over the 3-days of the experiment in the warm-adapted animals treated with the protein synthesis inhibitor. As the increase in mitochondrial GPAT activity in the cold-exposed animals treated with cycloheximide was very small in absolute terms, it is likely that changes in mitochondrial GPAT activity levels require synthesis of new enzyme and is not an activation of an existing pool of enzyme. The fact that the enzyme could not be purified in

the time available means that direct assessment of enzyme levels still needs to be addressed.

Studying the overall picture of the changes in the activities of the glycerolipid synthesis enzymes it is possible to suggest that in BAT the regulation of flux through the glycerolipid synthesis pathway may be controlled via hormonal regulation of GPAT. GPAT is the first committed enzyme and as such, an ideal enzyme to regulate flux. Also the variation in GPAT activity in BAT, especially in the mitochondrial form (35-fold from the lowest level in diabetics to the cold-exposed hypothyroid value), which is very rapid and highly reproducible indicates this as a regulatory point. The activities of the other enzymes in the pathway are higher than that of GPAT and changes in their activities small in comparison. Mitochondrial GPAT would appear to be the more likely regulator of flux through the glycerolipid synthesis pathway in cold-stressed tissue since it is localized with the predominant amount of FAS, which is required to activate fatty acids prior to their oxidation or esterification. The mitochondrial location also would be logical in that it would be ideal for channelling of enough fatty acyl-CoA away from oxidation to ensure supply of membrane phospholipids and replenish triacylglycerol stores as required. The increase in mitochondrial GPAT activity in BAT in physiological states where thermogenesis is inhibited and the tissue atrophied may suggest that glycerolipid synthesis in BAT is regulated in two ways, reflecting two different roles for the pathway. The first, as described above, would primarily involve supply of phospholipids for membrane production during tissue hypertrophy; the second would be storage of supplied fatty acids in times when they would not otherwise be oxidised (to produce heat), in other words a more white adipose tissue-like role. It is possible to suggest that the second of these roles would be regulated primarily through the microsomal enzymes which are thought to be primarily involved in triacylglycerol synthesis in other tissues.

What is very evident is that the hormonal signals involved in the regulation of glycerolipid synthesis in brown adipose tissue are complex, probably involving more than one hormonal signal and numerous second messenger pathways. Further investigation into the role of the adrenal hormones in the regulation of the cold-induced increases in the

glycerolipid synthesis enzymes, and the production of antibodies to GPAT and PPH, would provide the most interesting next steps in the study of this aspect of brown adipose tissue metabolism.

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